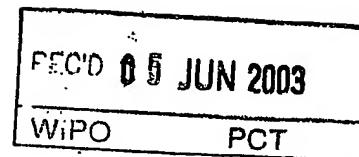


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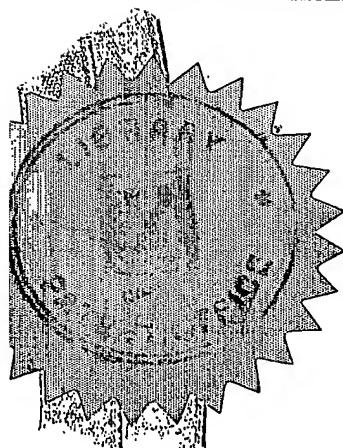
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Application for Patent

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I (Name and address of applicant, and in case of body corporate - place of incorporation)

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an invention the title of which is **תולדות של שרשרות גמא של הקולטן לאינטראקציון-2, הכנון והשימוש בהן**
(Hebrew)
(באנגלית)

rivatives of the IL-2 receptor gamma chain, their preparation and use

(באנגלית)

English)
 hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי יינתן עלייה פטנט

| Application of Division | | דרישת דין קדימה Priority Claim | | |
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**תולדות של שרeration גמא של הקולטן לאינטראקן-2, הכנתן
והשימוש בהן**

**Derivatives of the IL-2 receptor gamma chain, their
preparation and use**

Applicant: Yeda Research and Development Co. Ltd.

Ref: 530A

Inventors: Wallach David, Parameswaran Ramakrishnan

FIELD OF THE INVENTION

This invention relates to the use of fragments of the IL-2 γ chain receptor and NIK for modulation of NIK and signal activities controlled by NIK.

BACKGROUND OF THE INVENTION

Nuclear factors κ B (NF- κ B) is a family of inducible eukaryotic transcription factor complexes participating in regulation of immune response, cell growth, and survival [Ghosh et al. 1998]. The NF- κ B factors are normally sequestered in the cytoplasmic compartment by physical association with a family of cytoplasmic ankyrin rich inhibitors termed I κ B, including I κ B α and related proteins [Baldwin et al. 1996]. In response to diverse stimuli, including cytokines, mitogens, and certain viral gene products, I κ B is rapidly phosphorylated at serines 32 and 36, ubiquitinated and then degraded by the 26S proteosome, which allows the liberated NF- κ B to translocate to the nucleus and participate in target gene transactivation [Mercurio et al 1999, Pahl et al 1999]. Recent molecular cloning studies have identified a multi subunit I κ B kinase that mediates the signal-induced phosphorylation of I κ B. The IKK is composed of two catalytic subunits, IKK α and IKK β , and a regulatory subunit IKK γ . The catalytic activity of both IKK α and IKK β can be activated by a multitude of different NF- κ B inducers, including the inflammatory cytokines, tumor necrosis factor and interleukin-1, the T cell receptor and the T cell costimulatory protein, CD28 [Karin et al 2000].

NF- κ B-inducing kinase, NIK, (MAP3K14) is a mitogen activated protein kinase (MAP3K) that was discovered by applicants in 1996 (WO9737016) while screening for proteins that bind to the TNF-receptor associated adaptor protein TRAF2 [Rothe et al. 1994, Takeuchi et al. 1996]. Marked activation of NF- κ B upon overexpression of this protein kinase, and

effective inhibition of NF- κ B activation in response to a variety of inducing agents (LMP1, TNFR1, TNFR2, RANK, hTollR, CD3/CD28, interleukin-1R, human T-cell lymphotropic virus-1 Tax, LPS and others [Mlinin et al. 1997, Sylla et al 1998, Darnay et al. 1999, Lin et al. 1999, Geleziunas et al. 1998] upon expression of catalytically inactive NIK mutants suggested that NIK participates in signaling for NF- κ B activation [Mlinin et al. 1997].

Targeted disruption of the NIK gene [Yin et al 2001] and study of a naturally occurring mice strain with a point missense mutation in NIK (glycine to arginine at mNIK codon 855) [Shinkaura et al. 1999] revealed an essential role of NIK in lymphoid organ development, thus the mice mutant strain has been called 'alymphoplasia (aly)' mice. Both the *aly/aly* and NIK knockout mice manifest systemic absence of lymph nodes and Peyer's patches, disorganized splenic and thymic architectures, and immunodeficiency whose most resilient features are low serum Ig levels and lack of graft rejection [Shinkaura et al. 1999]. These abnormalities apparently reflect aberrant signaling by a variety of receptors. The developmental deficiencies of the NIK mutant mice resemble those found in mice deficient in the LT β receptor (LT β R) suggesting that NIK participates in signaling by this particular receptor. Impaired B cell proliferative capacity in the *aly/aly* mice could be shown to correlate to a deficient response of these cells to LPS and CD40L [Garceau et al. 2000], and presence of excessive amounts of B1 cells in the mice peritoneal cavity could be ascribed to defects in homing of peritoneal cells to the gut associated lymphatic tissue system as a consequence of deficient chemokine receptor signaling in the secondary lymphoid tissue [Fagarasan et al. 2000].

Apart from these and probably other contributions to the regulation of the development and function of the immune system, NIK seems also to be involved in the regulation of various non-immune functions. The *aly/aly* (though not the NIK knockout) mice display deficient mammary gland development [Miyawaki 1994]. Moreover, *in vitro* studies implicated NIK in signaling that leads to skeletal muscle cell differentiation [Canicio et al. 2001] and in the survival and differentiation of neurons [Foher et al 2000].

Consistent with the suggested role of NIK as mediator of NF- κ B activation, fibroblasts derived from *aly/aly* and NIK-/- mice fail to activate NF- κ B in response to LT β R activation. Moreover, LT β R upregulation of VCAM-1, which occurs through NF- κ B activation, is abnormal in *aly/aly* murine embryonic fibroblasts [Matsumoto et al. 1999]. Deficient phosphorylation of I κ B has also been noted in the response of *aly/aly* B-lymphocytes to CD40 ligation. In contrast, in dendritic cells of these mice CD40-induced phosphorylation of I κ B appeared normal [Garceau et al 1998]. *Aly/aly* peritoneal cells are also incapable of responding to the chemokine SLC with increased NF- κ B activity [Fagarasan et al. 2000]. However, in none of the cells examined so far was the effect of TNF or IL-1 on NF- κ B activation found to be ablated by NIK mutation.

Assessment of the pattern of the NF- κ B species in lymphoid organs of *aly/aly* mice indicated that, apart from its role in the regulation of NF- κ B complex(s) comprised of Rel proteins (A+p50) and I κ B, NIK also participates in controlling the expression/activation of other NF- κ B species. Most notably, the lymphocytes of the *aly/aly* mice were deficient of p52, an NF- κ B species that is specifically formed in mature B-lymphocytes through proteolytic processing of an inactive precursor, p100 (NF- κ B2), suggesting a deficiency in p100 – p52 conversion [Yamada et al. 2000]. Indeed, NIK has been shown to participate in site specific phosphorylation of p100. Both directly and through phosphorylation of IKK α which in turn phosphorylates p100. This phosphorylation serves as a molecular trigger for ubiquitination and active processing of p100 to form p52. This p100 processing activity was found to be ablated by the aly mutation [Xiao et al. 2001, Senftleben et al. 2001].

In view of the structural homology of NIK to MAP3Ks, some attempts have been made to explore the involvement of NIK in the three other main protein kinase cascades known to involve MAP3Ks (the MAP kinase cascades: the ERK, JNK and p38 cascades)[Akiba et al. 1998]. Though in certain cells NIK seems not to participate in any of these cascades, some other cells (PC12) do appear to involve NIK in the ERK cascade [Foher et al. 2000].

Evidence has also been presented that in certain cells NIK may participate in signaling to the phosphorylation of Jun, the downstream target of the JNK cascade, in a way that is independent of this particular cascade [Akiba et al. 1998, Natoli et al. 1997]. In all, these findings indicate that NIK indeed serves as a mediator of NF- κ B activation, but may also serve other functions, and that it exerts these functions in a cell- and receptor-specific manner.

Like other MAP3Ks, NIK can be activated as a consequence of phosphorylation of the 'activation loop' within the NIK molecule. Indeed, mutation of a phosphorylation-site within this loop (Thr-559) prevents activation of NF- κ B upon NIK overexpression [Lin et al. 1999]. In addition, the activity of NIK seems to be regulated through the ability of the regions upstream and downstream of its kinase motif to bind to each other. The C-terminal region of NIK downstream of its kinase moiety has been shown to be capable of binding directly to IKK α [Regnier et al. 1997] as well as to p100 [Xiao et al. 2001] and to TRAF2 [Malinin et al. 1997] these interactions are apparently required for NIK function in NF- κ B signaling. The N-terminal region of NIK contains a negative-regulatory domain (NRD), which is composed of a basic motif (BR) and a proline-rich repeat motif (PRR) [Xiao et al. 2000]. Apparently, the N-terminal NRD interacts with the C-terminal region of NIK in cis, thereby inhibiting the binding of NIK to its substrate (IKK α and p100). Ectopically expressed NIK seems to spontaneously form oligomers in which these bindings of the N-terminal to the C-terminal regions in each NIK molecule are apparently disrupted, and display a high level of constitutive activity [Lin et al. 1999]. The binding of the NIK C-terminal region to TRAF2 (as well as to other TRAF's) most likely participates in the activation process of NIK. However, its exact mode of participation is unknown.

There is likewise rather limited information yet of the downstream mechanisms in NIK action. Evidence has been presented that NIK, through the binding of its C-terminal region to IKK α can activate the I κ B kinase (IKK) complex. It has indeed been shown to be capable of phosphorylating serine-176 in the activation loop of IKK α and its activation thereby [Ling et al. 1998]. Consistently with such mode of action, studies of the mechanisms accounting to the deficient activation of NF- κ B by the LT β R in *aly/aly* mice murine embryonic fibroblasts

(MEF's) indicated that NIK mutation ablates activation of the IKK signalosome and the consequent phosphorylation of I κ B [Matsushima et al 2001]. These findings were not supported, however, by the analysis of MEF's derived from NIK $^{-/-}$ mice. Although the NIK deficient MEF's are unable to manifest NF- κ B activation in response to LT β , they do seem to respond normally to it in terms of I κ B phosphorylation and degradation [Yin et al. 2001]. According to these findings, NIK may not participate at all in the activation of the IKK complex by the LT β R but is rather involved by an as yet unknown mechanism in controlling the transcriptional action of the NF- κ B complex after its translocation to the nucleus. There are also still uncertainties as to the way by which NIK triggers p100 phosphorylation and processing. Its ability to bind p100 directly through its C-terminal region and phosphorylate it suggests that p100 serves as a direct NIK substrate [Xiao et al. 2000]. Nevertheless, a recent study has suggested that NIK mediates p100 phosphorylation in an indirect way, through phosphorylation and thus activation of IKK α that in turn phosphorylates p100 [Senftleben et al.2001].

Yamamoto and Gaynor reviewed the role of NF- κ B in pathogenesis of human disease (Yamamoto and Gaynor 2001). Activation of the NF- κ B pathway is involved in the pathogenesis of chronic inflammatory disease, such as asthma, rheumatoid arthritis (see Tak and Firesfein, this Perspective series, ref. Karin et al. 2000), and inflammatory bowel disease. In addition, altered NF- κ B regulation may be involved in other diseases such as atherosclerosis (see Collins and Cybulsky, this series, ref. Leonard et al. 1995) and Alzheimer's disease (see Mattson and Camandola, this series, ref. Lin et al. 1999), in which the inflammatory response is at least partially involved. Finally, abnormalities in the NF- κ B pathway are also frequently seen in a variety of human cancers.

Several lines of evidence suggest that NF- κ B activation of cytokine genes is an important contributor to the pathogenesis of asthma, which is characterized by the infiltration of inflammatory cells and the dysregulation of many cytokines and chemokines in the lung (Ling et al. 1998). Likewise, activation of the NF- κ B pathway also likely plays a role in the

pathogenesis of rheumatoid arthritis. Cytokines, such as TNF- α , that activate NF- κ B are elevated in the synovial fluid of patients with rheumatoid arthritis and contribute to the chronic inflammatory changes and synovial hyperplasia seen in the joints of these patients (Malinin et al. 1997). The administration of antibodies directed against TNF- α or a truncated TNF- α receptor that binds to TNF- α can markedly improve the symptoms of patients with rheumatoid arthritis.

Increases in the production of proinflammatory cytokines by both lymphocytes and macrophages have also been implicated in the pathogenesis of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (Matsumoto et al. 1999). NF- κ B activation is seen in mucosal biopsy specimens from patients with active Crohn's disease and ulcerative colitis. Treatment of patients with inflammatory bowel diseases with steroids decreases NF- κ B activity in biopsy specimens and reduces clinical symptoms. These results suggest that stimulation of the NF- κ B pathway may be involved in the enhanced inflammatory response associated with these diseases.

Atherosclerosis is triggered by numerous insults to the endothelium and smooth muscle of the damaged vessel wall (Matsushima et al. 2001). A large number of growth factors, cytokines, and chemokines released from endothelial cells, smooth muscle, macrophages, and lymphocytes are involved in this chronic inflammatory and fibroproliferative process (Matsushima et al. 2001). NF- κ B regulation of genes involved in the inflammatory response and in the control of cellular proliferation likely plays an important role in the initiation and progression of atherosclerosis.

Finally, abnormalities in the regulation of the NF- κ B pathway may be involved in the pathogenesis of Alzheimer's disease. For example, NF- κ B immunoreactivity is found predominantly in and around early neuritic plaque types in Alzheimer's disease, whereas mature plaque types show vastly reduced NF- κ B activity (Mercurio et al. 1999). Thus, NF- κ B activation may be involved in the initiation of neuritic plaques and neuronal apoptosis during

the early phases of Alzheimer's disease. These data suggest that activation of the NF- κ B pathway may play a role in a number of diseases that have an inflammatory component involved in their pathogenesis.

In addition to a role in the pathogenesis of diseases characterized by increases in the host immune and inflammatory response, constitutive activation of the NF- κ B pathway has also been implicated in the pathogenesis of some human cancers. Abnormalities in the regulation of the NF- κ B pathway are frequently seen in a variety of human malignancies including leukemias, lymphomas, and solid tumors (Miyawaki et al. 1994). These abnormalities result in constitutively high levels of NF- κ B in the nucleus of a variety of tumors including breast, ovarian, prostate, and colon cancers. The majority of these changes are likely due to alterations in regulatory proteins that activate signaling pathways that lead to activation of the NF- κ B pathway. However, mutations that inactivate the I κ B proteins in addition to amplification and rearrangements of genes encoding NF- κ B family members can result in the enhanced nuclear levels of NF- κ B seen in some tumors.

Consistent with the suggested role of NIK as mediator of NF- κ B activation there exists a need for a modulator of NIK activity for preventing or alleviating diseases in which NIK and NF- κ B are involved.

IL2 is a protein of 133 amino acids (15.4 kDa) with a slightly basic pI. It does not display sequence homology to any other factors. Murine and human IL2 display a homology of approximately 65 percent. IL2 is synthesized as a precursor protein of 153 amino acids with the first 20 aminoterminal amino acids functioning as a hydrophobic secretory signal sequence. The protein contains a single disulfide bond (positions Cys58/105) essential for biological activity.

Mouse and human IL2 both cause proliferation of T-cells of the homologous species at high efficiency. Human IL2 also stimulates proliferation of mouse T-cells at similar

concentrations, whereas mouse IL2 stimulates human T-cells at a lower (sixfold to 170-fold) efficiency.

IL2 is a growth factor for all subpopulations of T-lymphocytes. The IL2R-alpha receptor subunit is expressed in adult T-cell leukemia (ATL). Since freshly isolated leukemic cells also secrete IL2 and respond to it IL2 may function as an autocrine growth modulator for these cells capable of worsening ATL.

IL2 also promotes the proliferation of activated B-cells also this requires the presence of additional factors, for example, IL-4. In vitro IL2 also stimulates the growth of oligodendroglial cells.

Due to its effects on T-cells and B-cells IL2 is a central regulator of immune responses. It also plays a role in anti-inflammatory reactions in hematopoiesis and in tumor surveillance. IL2 stimulates the synthesis of IFN-gamma in peripheral leukocytes and also induces the secretion IL-1 TNF-alpha and TNF-beta..

The biological activities of IL2 are mediated by a membrane receptor that is expressed almost exclusively on activated, but not on resting, T-cells. Activated B-cells and resting mononuclear leukocytes rarely express this receptor.

Three different types of IL2 receptors are distinguished that are expressed differentially and independently. The high affinity IL2 receptor constitutes approximately 10 percent of all IL2 receptors expressed by a cells. This receptor is a membrane receptor complex consisting of the two subunits IL2R-alpha and IL2R-beta as the ligand binding domains and a gamma chain as a signaling component. IL2R-beta is expressed constitutively on resting T-lymphocytes, NK-cells, and a number of other cell types while the expression of IL2R-alpha is usually observed only after cell activation. IL2R-alpha is, however, synthesized constitutively by a number of tumor cells and by HTLV-1-infected cells.

IL2 receptor expression of monocytes is induced by IFN γ so that these cells become tumor-cytotoxic.

A third 64 kDa subunit of the IL2 receptor, designated gamma, has been described recently. Murine and human gamma subunits of the receptor have approximately 70 percent sequence identity at the nucleotide and amino acid levels. This subunit is required for the generation of high and intermediate affinity IL2 receptors but does not bind IL2 by itself. These two receptor types consist of a alpha-beta-gamma heterotrimer and a beta-gamma heterodimer, respectively. The gene encoding the gamma subunit of the IL2 receptor maps to human chromosome Xq13, spans approximately 4.2 kb and contains eight exons. Relationships to markers in linkage studies suggest that this gene and SCIDX1, the gene for X-linked severe combined immunodeficiency, have the same location. Moreover, in each of 3 unrelated patients with X-linked SCID, a different mutation in the IL2R-gamma gene has been observed.

The levels of sIL2R appear to correlate with the severity of HIV-induces diseases and may be of diagnostic value also in other settings.

X-linked severe combined immunodeficiency (XSCID) is a rare and potentially fatal disease caused by mutations of IL2RG, the gene encoding the IL-2R γ chain, a component of multiple cytokine receptors that are essential for lymphocyte development and function (Noguchi et al. 1993). To date, over 100 different mutations of IL2RG resulting in XSCID have been published.

The IL-2R γ chain is a subunit of the IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21 receptor complexes wherefore it now dubbed as the 'common γ chain' (cyc).

The IL-2R γ chain receptor DNA-sequence, a vector possessing said DNA-sequence, a cell transformed with said vector, a method for the production of an IL-2 receptor gamma chain and an antibody to an IL-2 receptor gamma chain molecule are described in EP0578932. EP0578932 relates to the whole common gamma chain and especially to the extracellular N-terminal domain.

Recent gene knock out studies indicate a pivotal role of the cyc in lymphopoiesis [DiSanto et al 1995].

SUMMARY OF THE INVENTION

The present invention relates to the use of a polypeptide comprising the intracellular domain of the IL-2 receptor common gamma chain (cyc) or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, or a vector encoding such polypeptide or a specific intrabody in the manufacture of a medicament for treatment of a disease in which NIK or NF- κ B activation is involved in its pathogenesis e.g. cancer or diseases resulting from excessive immune responses e.g. and arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.

The invention also provides methods for treatment and / or prevention of diseases resulting from excessive immune responses or diseases in which NIK or NF- κ B activation is involved, comprising administration of an effective amount of a polypeptide comprising the intracellular domain of the IL-2 receptor common gamma chain (cyc) or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof or a vector comprising a sequence encoding such polypeptides, or a specific intrabody.

Moreover, the present invention relates to the use of a polypeptide comprising the cyc binding domain in NIK (SEQ ID NO:18) or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, or a vector encoding such polypeptide or a specific intrabody in the manufacture of a medicament for treatment of a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.

The invention further provides methods for treatment and / or prevention of diseases, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease, comprising administration of an effective amount of a

polypeptide of NIK (SEQ ID NO:18) or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof or a vector encoding such polypeptides, or a specific intrabody.

In another aspect, the present invention relates to a polypeptide comprising the intracellular domain of the IL-2 γ chain receptor, or a mutein, fusion protein, functional derivative, active fraction, circularly permuted derivative or fragment thereof. More specifically the invention relates to a polypeptide comprising the amino acid sequence in SEQ ID NO: 1, encoded by the nucleotide sequence in SEQ ID NO: 5, preferably to a polypeptide comprising the amino acid sequence in SEQ ID NO: 2 encoded by the nucleotide sequence in SEQ ID NO: 6 and more preferably the amino acid in SEQ ID NO: 3.

Furthermore, the present invention provides a fragment of NIK corresponding to the cyc binding site (SEQ ID NO:18).

In addition the invention relates to a DNA encoding a polypeptide according to the invention, preferably fused to the signal peptide e.g. that of the growth hormone and to a vector comprising said DNA sequence capable of expressing the encoded polypeptide in an appropriate host cell.

In one embodiment a method is provided for producing a polypeptide according to the invention, comprising introducing said vector into a prokaryotic or eukaryotic host cell, preferably CHO, cultivating the cell and isolating the polypeptide produced.

The invention also provides a vector comprising the sequence encoding the polypeptide of the invention for the manufacture of a medicament for the treatment of diseases in which NIK and NF- κ B activity is involved, e.g. cancer and for the treatment and/or prevention of diseases resulting from excessive immune responses e.g. asthma, rheumatoid arthritis, inflammatory bowel disease, Alzheimer's disease.

In another aspect the invention provides the use of a polypeptide of the invention for designing organic molecules which interfere in binding of cyc to NIK.

In another aspect the invention provides a method for screening for molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction, and the organic molecules identified.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of cyc domains and the residues in which stop codons were introduced to generate deletion mutants. L-Leader 1-23, ECD-extra cellular domain, TM-trans membrane domain, ICD- intra cellular domain.

Figure 2 shows results on NIK-cyc interaction in mammalian cells monitored by immunoprecipitation assay. Western blot analysis were detected with anti cyc antibody of the following samples: 1- lysates of 293-T cells transfected with pcDNA3cyc and immunoprecipitated with anti cyc, 2- lysates of 293-T cells transfected with pcDNA3cyc and immunoprecipitated with anti NIK antibody. 6- lysates of 293-T cells transfected with pcS3MTNIK (expressing myc tagged NIK) and pcDNA3cyc and NIK immunoprecipitated with anti myc antibody. 3- the same as in 6 only that the pcS3MTNIK is exchanged with the pcS3MTNIKaly. 4- lysates of 293-T cells transfected with pcdnNIK (C-terminal domain of NIK residues 624-947) and immunoprecipitated with anti cyc antibody and 5- lysates of 293-T cell transfected with pcdnNIK and pcDNA3cyc immunoprecipitated with anti NIK antibodies (not anti myc antibody as in 3 and 6).

Figure 3 shows results on NIK-cyc interaction in mammalian cells monitored by immunoprecipitation assay. Western blot analysis of immunoprecipitates and total 293-T cell lysates detected with anti NIK antibody. The samples analysed were the following:

3- lysate of cells transfected with pcS3MTNIK immunoprecipitated with anti NIK. 2- lysate of cells transfected with pcS3MTNIK and immunoprecipitated with anti cyc antibody. 1-

lysates of cells transfected with both pcS3MTNIK and pcDNA3cyc and immunoprecipitated with anti cyc antibody. 5- lysate of non-transfected cells. 4 and 6- lysates of cells transfected with both pcS3MTNIK and pcDNA3cyc or cells transfected with pcS3MTNIK alone, respectively, before immunoprecipitation.

Figure 4 shows the concentration-dependent effect of cyc on NIK induced NF- κ B activation. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in 293-T cells was induced by overexpressing NIK. Luciferase expression was monitored in cells transfected with the following plasmids: empty plasmid (sample pc), empty plasmid and a plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3luciferase, 0.5 μ g/well) (sample pc+luc), 1 μ g pcS3MTNIK and pcDNA3luciferase (sample NIK 1mcg), 1 μ g pcS3MTNIK, 0.1 μ g/well pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc 0.1mcg), 1 μ g pcS3MTNIK, 0.5 μ g/well pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc 0.5 mcg), and 1 μ g pcS3MTNIK with 1 μ g/well pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc 1 mcg).

Figure 5 shows the effect of a dominant negative mutant of NIK (dnNIK, residues 624-947) on cyc enhanced NF- κ B activation. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in 293-T cells was induced by overexpressing NIK. Luciferase expression was monitored in cells transfected with the following plasmids: empty plasmid (sample pc), empty plasmid and a plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3luciferase) (sample pc+luc), pcS3MTNIK and pcDNA3luciferase (sample NIK), pcS3MTNIK, pcS3MTdnNIK and pcDNA3luciferase (sample NIK+dnNIK) pcDNA3cyc and pcDNA3luciferase (sample cgc), pcS3MTNIK, pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc), pcS3MTdnNIK, pcDNA3cyc and pcDNA3luciferase (sample cgc+dnNIK), pcS3MTNIK, pcDNA3cyc, pcS3MTdnNIK and pcDNA3luciferase (sample NIK+cgc+dnNIK). pcS3MTdnNIK, pcS3MTNIK and pcDNA3cyc were used at a concentration of 1, 1, and 0.1 μ g/well respectively.

Figure 6 shows the effect of cyc on NF- κ B activation induced by the NIK Δ ly mutant. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in cells is induced by overexpressing NIK. Luciferase expression was monitored in 293-T cells transfected with the following plasmids: empty plasmid (sample pc), empty plasmid and a plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3luciferase) (sample pc+luc), pcS3MTNIK and pcDNA3luciferase (sample NIK), pcDNA3luciferase and pcDNA3cyc (sample cgc), pcS3MTNIK, pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc), 1 μ g pcS3MT Δ lyNIK and pcDNA3luciferase (sample alyNIK) and pcS3MT Δ lyNIK, pcDNA3cyc and pcDNA3luciferase (sample alyNIK+cgc). pcS3MT Δ lyNIK, pcS3MTNIK and pcDNA3cyc were used at a concentration of 1, 1, and 0.1 μ g/well respectively.

Figure 7 shows the effect of a 41 amino acid polypeptide derived from the membrane distal end of cyc (41MDD) on NIK induced NF- κ B activation and enhancement by full-length cyc. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in 293-T cells was induced by overexpressing NIK. Enhancement of NF- κ B induction is obtained by overexpressing NIK and expressing the full cyc at low concentration. Luciferase expression was monitored in cells transfected with the following plasmids: empty plasmid (sample pc), empty plasmid and a plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3luciferase) (sample pc+luc), pcS3MTNIK and pcDNA3luciferase (sample NIK), pcS3MTNIK, pcDNA3cyc and pcDNA3luciferase (sample NIK+cgc), pcS3MTNIK, a plasmid expressing GST (pGST) and pcDNA3luciferase (sample NIK+GST), pcS3MTNIK, pcDNA3cyc, pcGST and pcDNA3luciferase (sample NIK+GST+cgc), pcS3MTNIK, pcDNA3cyc, pcGST-41MDD and pcDNA3luciferase (sample NIK+cgc+41GST). The plasmids pcS3MTNIK, pcDNA3cyc, pcGST-41MDD and pcDNA3luciferase were used at concentrations of 0.5, 0.05, 2 and 0.5 μ g/ml respectively.

Figure 8 shows the effect of *cyc* deletion mutants, deleted at the C-terminal end of the protein, and on NIK induced NF- κ B activation. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in Hela cells was induced by overexpressing NIK. Luciferase expression was monitored in cells transfected with the following plasmids: empty plasmid and a plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3luciferase) (sample pc+luc), pcS3MTNIK and pcDNA3luciferase (sample NIK), pcS3MTNIK, pcDNA3cyc and pcDNA3luciferase (sample NIK +cgc), pcS3MTNIK, pcDNA3cyc357 and pcDNA3luciferase (sample NIK+1-357), pcS3MTNIK, pcDNA3cyc341 and pcDNA3luciferase (sample NIK+1-341), pcS3MTNIK, pcDNA3cyc325 and pcDNA3luciferase (sample NIK+1-325) and pcS3MTNIK, pcDNA3cyc303 and pcDNA3luciferase (sample NIK+1-303). Plasmids pcS3MTNIK, pcDNA3cyc/deleted, pcDNA3luciferase, were all used at the same concentration of 0.5 μ g/ml. Total amount of DNA used was normalized with empty plasmid pcDNA3.

Figure 9 shows the effect of *cyc* on the in vitro kinase activity of NIK. 293-T cells were transfected with 10 μ g pcDNA3cyc (Line 1), 10 μ g pcDNA3cyc and 10 μ g of pcS3MTNIK (Line 2), 10 μ g of pcS3MTNIK (Line 3) or 10 μ g of pcS3MTNIK and 10 μ g of a plasmid encoding the kinase IKK1 (pIKK1) (Line 4). 24 hours later, cells were harvested, lysed and immunoprecipitation was carried out with rabbit anti NIK antibody pre adsorbed to protein A sepharose beads. Kinase reaction was performed with 5 μ ci ATP γ as previously described (Uhlik et al. 1998).

Figure 10 shows the effect of overexpression of full ICD *cyc* or its 41 amino acid membrane distal domain on NF- κ B activation induced via the LT β receptor. Activation of NF- κ B is monitored by the luciferase reporter assay (for details see Example 10). NF- κ B activation in mouse embryonic fibroblast cells was induced with LT β . Luciferase expression was monitored in cells transfected with the following plasmids: empty plasmid (sample pc), a plasmid expressing GST (pcGST) and pcDNA3luciferase (sample pcGST+luc), a plasmid encoding the GST fusion protein with the intracellular domain of *cyc* (pGSTICcyc) and

pcDNA3luciferase (sample GSTICcgc+*luc*) and a plasmid encoding the GST fusion with the 41 polypeptide from the membrane distal domain of *cyc* (pGST41MDD) and pcDNA3luciferase (sample GST-41MDD+*luc*). Plasmids pGSTICcyc, pGST41MDD, and were used at 1 μ g/well and pcDNA3luciferase were used at a concentration of 0.5 μ g/well. Empty plasmid, pcDNA3 was used as a carrier to normalize the total DNA concentration to 2 μ g/well. The levels of luciferase activity are expressed in relative light units (RLU).

Figure 11 shows the amino acid sequence of the intracellular domain of *cyc*.

Figure 12 shows the amino acid sequence of the 41 amino acid polypeptide from the membrane distal domain of *cyc* (41MDD).

Figure 13 shows the nucleotide sequence of the intracellular domain of *cyc* (cycICD).

Figure 14 shows the nucleotide sequence of the 41 polypeptide from the membrane distal domain of *cyc* (41MDD).

Figure 15 shows the sequence of 12 aminoacids at the C-terminus of *cyc* involved in binding NIK.

Figure 16A shows interaction of endogenous NIK and *cyc*. Periferal blood mononuclear cells (PMBC) (500×10^6 cells) were incubated either in the presence of IL-2, lysed and immunoprecipitated (IP) with anti *cyc* antibodies (for immunoprecipitation see Example 9). Co-immunoprecipitated proteins bound to *cyc* were detected in Western blots (WB) using relevant antibodies. The antibodies used in WB to detect co-immunoprecipitation with *cyc* were ANTI-NIK, ANTI-IKK α (IKK-1), ANTI-IKK β (IKK2), and ANTI-IKK γ (NEMO). The co-immunoprecipitated proteins were in lysates of cells tested at 0 and up to four-hour

incubation with IL-2. A non-relevant IgG was used for immunoprecipitation (IP) as the control.

Figure 16B shows that the signalosome co-immunoprecipitated with cyc is active. The immunoprecipitates prepared as in Figure 16A were tested in a kinase assay (see details on Example 11) using GST- $\text{IKB}\alpha 1-54$ as a substrate for phosphorylation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of fragments of NIK and/or of IL-2 common gamma chain (cyc) for the inhibition of cyc-NIK interaction. Such inhibition will allow treatment or prevention of a disease in which the activity of NIK is involved in its pathogenesis e.g. by administration of a pharmaceutical composition comprising such an inhibitor.

The fragments of cyc, according to the invention, comprise the cytoplasmic intracellular domain of the IL-2 γ chain receptor (ICDcyc) SEQ ID NO: 1 or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

The preferred fragments of the intracellular cyc, which were found to bind NIK, comprise the 44 amino acid residues at the membrane proximal domain (44MPD) (from residue 282 to 325), SEQ ID NO: 17 and the 41 amino acid residues at the membrane distal domain (41MDD) (from residues 329 to 369), SEQ ID NO: 2 and (Figure 12) and to their mutein, fusion protein, functional derivative, active fraction, circularly permuted derivative or fragment thereof.

The preferred fragment of NIK, which was found to bind cyc (NIK640-720), comprises the 81 amino acid residues at the C-terminus from residue 640-720, SEQ ID NO: 18 or a

mutein, fusion protein, functional derivative, active fraction, circularly permuted derivative or fragment thereof.

The invention relates to ICDcyc, 44MPD, 41MDD and NIK640-720 and to their salts, functional derivatives, precursors and active fractions as well as its active mutants, i.e. other proteins or polypeptides wherein one or more amino acids of the structure are eliminated or substituted by other amino acids or one or more amino acids were added to that sequence in order to obtain polypeptides or proteins having the same activity of the ICDcyc, 44MPD, 41MDD and NIK640-720 and comprises also the corresponding "fusion proteins" i.e. polypeptides comprising the ICDcyc, 44MPD, 41MDD, and NIK640-720 or a mutation thereof fused with another protein and having a longer lasting half-life in body fluids. The ICDcyc, 44MPD, 41MDD and NIK640-720 can therefore be fused with another protein such as, for example, an immunoglobulin.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the ICDcyc, 44MPD, 41MDD and the NIK640-720 protein of the invention or muteins thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulphuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar activity to the ICDcyc, 44MPD, 41MDD and NIK640-720 protein of the invention or its muteins.

The definition "functional derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the terminal N- or C- groups according to known methods and are comprised in the invention when they are pharmaceutically acceptable i.e. when they do not destroy the protein activity or do not impart toxicity to the pharmaceutical compositions containing them. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyle- or aroyl-groups.

"Fragment" of the protein the present invention refers to any fragment or precursor of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same activity of the ICDcyc, 44MPD, 41MDD and NIK640-720 as medicament. The "circularly permuted derivative" may be e.g. compounds, which are converted into the ICDcyc, 44MPD, 41MDD and NIK640-720 in the human or animal body.

The terms in the present specification "polypeptide and protein" are interchangeable.

The present invention also concerns muteins of the above the ICDcyc, 44MPD, 41MDD and NIK640-720 protein of the invention, which muteins retain essentially the same biological activity of the ICDcyc, 44MPD, 41MDD and NIK640-720 protein having essentially only the naturally occurring sequences of the ICDcyc, 44MPD, 41MDD and NIK640-720. Such "muteins" may be ones in which up to about 20 and 10 amino acid residues may be deleted, added or substituted by others in the ICDcyc, 44MPD, 41MDD and NIK640-720 protein respectively, such that modifications of this kind do not substantially change the biological activity of the protein mutein with respect to the protein itself.

These muteins are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable thereof.

Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of the basic the ICDcyc, 44MPD, 41MDD and NIK640-720 such as to have substantially similar activity thereto. Thus, it can be determined whether any given mutein has substantially the same activity as the basic protein of the invention by means of routine experimentation comprising subjecting such a mutein to the biological activity tests set forth in Examples below.

Muteins of the ICDcyc, 44MPD, 41MDD and NIK640-720 protein which can be used in accordance with the present invention, or nucleic acid coding thereof, include a finite set of substantially the ICDcyc, 44MPD, 41MDD and NIK640-720 corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and

guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978; and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see. See Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publications and Wiley Interscience, New York, NY, 1987-1995; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Preferred changes for muteins in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of those in the protein having essentially the naturally-occurring the ICDcyc, 44MPD, 41MDD and NIK640-720 sequences, may include synonymous amino acids within a group, which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule, see Grantham, *Science*, Vol. 185, pp. 862-864 (1974). It is clear that insertions and deletions of amino acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under 20, and preferably under 10 for ICDcyc, and NIK640-720 and under 10 and preferable under 5 for 41MDD and 44MPD and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues, Anfinsen, "Principles That Govern The Folding of Protein Chains", *Science*, Vol. 181, pp. 223-230 (1973). Muteins produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table I. More preferably, the synonymous amino acid groups are those defined in Table II; and most preferably the synonymous amino acid groups are those defined in Table III.

TABLE I Preferred Groups of Synonymous Amino Acids

| Amino Acid | Synonymous Group |
|------------|-----------------------------------|
| Ser | Ser, Thr, Gly, Asn |
| Arg | Arg, Gln, Lys, Glu, His |
| Leu | Ile, Phe, Tyr, Met, Val, Leu |
| Pro | Gly, Ala, Thr, Pro |
| Thr | Pro, Ser, Ala, Gly, His, Gln, Thr |
| Ala | Gly, Thr, Pro, Ala |
| Val | Met, Tyr, Phe, Ile, Leu, Val |
| Gly | Ala, Thr, Pro, Ser, Gly |
| Ile | Met, Tyr, Phe, Val, Leu, Ile |
| Phe | Trp, Met, Tyr, Ile, Val, Leu, Phe |
| Tyr | Trp, Met, Phe, Ile, Val, Leu, Tyr |
| Cys | Ser, Thr, Cys |
| His | Glu, Lys, Gln, Thr, Arg, His |
| Gln | Glu, Lys, Asn, His, Thr, Arg, Gln |
| Asn | Gln, Asp, Ser, Asn |
| Lys | Glu, Gln, His, Arg, Lys |
| Asp | Glu, Asn, Asp |
| Glu | Asp, Lys, Asn, Gln, His, Arg, Glu |
| Met | Phe, Ile, Val, Leu, Met |
| Trp | Trp |

TABLE II More Preferred Groups of Synonymous Amino Acids

| Amino Acid | Synonymous Group |
|------------|-------------------------|
| Sers | Sers |
| Arc | His, Lys, Arg |
| Leu | Ile, Phe, Met, Leu |
| Pro | Ala, Pro |
| Thr | Thr |
| Ala | Pro, Ala |
| Val | Met, Ile, Val |
| Gly | Gly |
| Ile | Ile, Met, Phe, Val, Leu |
| Phe | Met, Tyr, Ile, Leu, Phe |
| Try | Phi, Try |
| Cys | Ser, Cys |
| His | Arg, Gln, His |
| Gln | Glu, His, Gln |
| Asn | Asp, Asn |
| Lys | Arg, Lys |
| Asp | Asn, Asp |
| Glu | FLN, Glu |
| Met | Phe, Ile, Val, Leu, Met |
| Trp | Trp |

TABLE III Most Preferred Groups of Synonymous Amino Acids

| Amino Acid | Synonymous Group |
|------------|------------------|
| Sers | Sers |
| Arc | Arc |
| Leu | Ile, Met, Leu |
| Pro | Pro |
| Thr | Thar |
| Alan | Alan |
| Val | Val |
| Gly | Gly |
| Ilea | Ile, Met, Leu |
| Phi | Phi |
| Try | Tyr |
| Cys | Ser, Cys |
| His | His |
| Gln | Gln |
| Asn | Asn |
| Lys | Lys |
| Asp | Asp |
| Glu | Glu |
| Met | Ile, Leu, Met |
| Trp | Trp |

Examples of production of amino acid substitutions in proteins which can be used for obtaining muteins of the protein for use in the present invention include any known method steps, such as presented in US patents RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins presented in US patent No. 4,904,584 (Straw et al).

In another preferred embodiment of the present invention, any mutein of the ICDcyc, 44MPD, 41MDD and NIK640-720 protein for use in the present invention has an amino acid sequence essentially corresponding to that of the above noted the ICDcyc, 44MPD, 41MDD and NIK640-720 protein of the invention. The term "essentially corresponding to" is intended to comprehend muteins with minor changes to the sequence of the basic protein which do not affect the basic characteristics thereof, particularly insofar as its ability to the ICDcyc, 44MPD, 41MDD and NIK640-720 is concerned. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding the ICDcyc, 44MPD, 41MDD and NIK640-720 protein of the invention, resulting in a few minor modifications, and screening for the desired activity in the manner discussed above (i.e inhibition of NIK-cyc interaction).

The present invention also encompasses the ICDcyc, 44MPD, 41MDD and NIK640-720 variants. A preferred ICDcyc, 44MPD, 41MDD and NIK640-720 variant are the ones having at least 80% amino acid identity, a more preferred the ICDcyc, 44MPD, 41MDD NIK640-720 variant is one having at least 90% identity and a most preferred variant is one having at least 95% identity to the ICDcyc, 44MPD, 41MDD and NIK640-720 amino acid sequence (SEQ ID Nos: 1, 2, 17 and 18 respectively).

The term "sequence identity" as used herein means that the amino acid sequences are compared by alignment according to Hanks and Quinn (1991) with a refinement of low homology regions using the Clustal-X program, which is the Windows interface for the ClustalW multiple sequence alignment program (Thompson et al., 1994). The Clustal-X program is available over the internet at <ftp://ftp-igbmc.u-strasbg.fr/pub/clustalx/>. Of course, it should be understood that if this link becomes inactive, those of ordinary skill in the art could find versions of this program at other links using standard internet search techniques without undue experimentation. Unless otherwise specified, the most recent version of any program referred herein, as of the effective filing date of the present application, is the one, which is used in order to practice the present invention.

Another method for determining "sequence identity" is the following. The sequences are aligned using Version 9 of the Genetic Computing Group's GDAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

Mutants in accordance with the present invention include those encoded by a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA under stringent conditions and which encodes the ICDcyc, 44MPD, 41MDD and NIK640-720 protein in accordance with the present invention, comprising essentially all of the naturally-occurring sequences encoding the ICDcyc, 44MPD, 41MDD and NIK640-720. For example, such a hybridizing DNA or RNA may be one encoding the same protein of the invention having, for example, the sequences set forth in Figs. 13 and 14 (SEQ ID: N5 and 6 encoding ICDcyc and 41MDD respectively), and sequences which may differ in its nucleotide sequence from the naturally-derived nucleotide sequence by virtue of the degeneracy of the genetic code, i.e., a somewhat different nucleic acid sequence may still code for the same amino acid sequence, due to this degeneracy.

The term "hybridization" as used herein shall include any process by which a strand of nucleic acid joins with complementary strand through a base pairing (Coombs J, 1994, Dictionary of Biotechnology, Stokton Press, New York NY). "Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (Dieffenbach and Dveksler, 1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

"Stringency" typically occurs in a range from about $T_m - 5^\circ\text{C}$ (5°C below the melting temperature of the probe) to about 20°C to 25°C below T_m .

The term "stringent conditions" refers to hybridization and subsequent washing conditions, which those of ordinary skill in the art conventionally refer to as "stringent". See Ausubel et al., Current Protocols in Molecular Biology, Greene Publications and Wiley Interscience, New

York, NY, 1987-1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

As used herein, stringency conditions are a function of the temperature used in the hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for determining the stability of hybrids of 100% identity expressed as melting temperature T_m of the DNA-DNA hybrid:

$$T_m = 81.5 C + 16.6 (\text{Log}M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1 C that the T_m is reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the T_m used for any given hybridization experiment at the specified salt and formamide concentrations is 10 C below the T_m calculated for a 100% hybrid according to the equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch.

As used herein, "highly stringent conditions" are those which provide a T_m which is not more than 10 C below the T_m that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. "Moderately stringent conditions" are those, which provide a T_m , which is not more than 20 C below the T_m that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. Without limitation, examples of highly stringent (5-10 C below the calculated or measured T_m of the hybrid) and moderately stringent (15-20 C below the calculated or measured T_m of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS (sodium dodecyl sulfate) at the appropriate temperature below the calculated T_m of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those, which allow less stable hybrids to form along with stable hybrids. The wash

conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE (standard saline-phosphate-EDTA), 5 X Denhardt's reagent, 0.5% SDS, 100 μ g/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20 to 25 C below the Tm. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 1987, 1999).

The invention is based on the finding that cyc and NIK interact, and that this interaction has an effect on the activity of NIK. The interaction was observed using a C-terminal fragment of NIK as bait in a two-hybrid screen of a bone marrow cDNA library. This interaction was confirmed by co-immunoprecipitation studies carried out in lysates of mammalian cells overexpressing NIK and cyc and also by co-immunoprecipitation studies in cells naturally expressing NIK and cyc. These studies revealed that cyc is efficiently co-precipitated with either the C-terminus of NIK (the same fragment used as the bait in the two hybrid system in which the cyc was identified) or with the full length of NIK.

The interaction of endogenous NIK and cyc was demonstrated in mononuclear cells. It was found that in mononuclear cells NIK is constitutively associated with cyc and upon IL-2 induction the signalosome components IKK-1, IKK-2, and IKK-3 are recruited to the IL-2 receptor through cyc. The IL-2 receptor common γ chain was found to bind to NIK at a different location, other than IKK-1 binding region. Therefore, the interaction of NIK and the signalosome components with IL-2 receptor common gamma chain is needed for signalling of IL-2. Therefore inhibition of the interaction of IL-2 common gamma chain and NIK may inhibit IL-2 signal. Thus, the NIK binding domain NIK640-720 or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof can be used to inhibit cyc-NIK interaction and therefore inhibit IL-2 signaling. Moreover, NIK640-720 can be used to inhibit any disease in which activation of a cytokine, having the common gamma chain in their receptors such as IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21, is involved in its pathogenesis.

The signalosome co-immunoprecipitated with cyc upon IL-2 stimulation was shown to be active in a kinase assay. Thus these results demonstrate that under physiological conditions, binding of endogenous cyc to NIK occurs, and that this interaction is involved in NIK activity and in NIK dependent NFkB activation. Therefore inhibiting the interaction of cyc and NIK will inhibit NFkB activation.

Proteins, polypeptides or organic molecules that inhibit the NIK/cyc interaction and therefore NIK action, can be used as drugs for treating a disease which is caused or aggravated by excessive NIK activity. For example, such inhibitor polypeptide may be the exact domains in the two proteins at which they bind to each other.

Multiple deletion mutants of both cyc and NIK were generated to define the binding domains in both proteins. The interactions were tested by yeast 2 hybrid tests and also by immunoprecipitation studies. The results obtained with the different deletion mutants indicate that a membrane distal domain of 41 residues spanning amino acids 329-369 (41MDD) and also a membrane proximal domain of 44 amino acids located between residues 282 and 325 (44MPD) of cyc are involved in binding to NIK. The binding of the 41MDD and 44MPD to NIK was analysed by the two-hybrid method. The binding of 41MDD to full length NIK or C-terminus NIK was tested in both orientations (i.e. 41MDD as the prey and NIK as the bait and vice versa). The interaction is relatively weak when NIK serves as the prey partner, but strong when NIK serves as the bait. The interaction of the 41 distal domain is stronger with the C-terminus of NIK than with the full length NIK. When 12 amino acids at the end of cyc (cyc residues 358-369, Fig 15 SEQ ID: NO 3 nucleotide sequence in SEQ ID NO: 4) were deleted from the cycICD, the binding to NIK decreases by 50% indicating that these residues play a major role in binding.

Mutagenesis was carried out in residues located within the 41MDD, to define the specific amino acids interacting with NIK. The interaction of proline rich motifs in signaling proteins with their cognate domains is well documented (Kay BK, Williamson MP, Sudol M. FASEB J 2000 Feb 14 (2): 231-421), 20% of the amino acids in the membrane distal 41 amino acids of cyc are prolines. Therefore, two consecutive prolines were mutated to alanine at two different sites within the 41 membrane distal amino acids of cyc: 1- PP 336,337AA and 2- PP360, 361AA and the effect of the mutation on binding of NIK tested by the two hybrid

assay. The results obtained of cyc mutagenesis demonstrate that the prolines at residues 360 and 361 are important for the binding to NIK. Thus the muteins of the present invention retains prolines at residues 360 and 361.

A domain in NIK, of 81 amino acid residues from 640-720 (NIK 640-720) was found to bind cyc.

cyc and NIK interaction was shown to be functionally significant. Reporter gene assays showed that cyc modulates NIK-induced NF- κ B activation. The effect of cyc depends on its concentration relative to NIK e.g. if cyc is present in a cell at lower concentrations than NIK it potentiates its effect (i.e. it enhances NF- κ B activation) while if present at equal or higher concentration it inhibits NIK's activity. Studies carried out with a dominant negative mutant of NIK showed that the NF- κ B enhancing activity of cyc is specifically exerted via NIK.

The effect of 41MDD on NF- κ B activation induced by NIK was also evaluated using reporter assays. The result shows that 41MDD, similar to the whole cyc, inhibits NF- κ B activation induced by NIK when present at higher or equal concentrations relative to NIK.

Therefore, the effect of ICDcyc and 41MDD administration may depend on the concentration of these proteins relative to NIK e.g. $[ICDcyc]$ or $[41MDD]/[NIK] \geq 1$ may result in inhibition of NF- κ B wherein $[ICDcyc]$ or $[ICDcyc]/[NIK] < 1$ may result in NF- κ B activation.

The fragment of cyc, 41MDD and/or 44MPD and/or NIK640-720 may serve as a candidate for peptide based drug designing. Organic molecules, based on the structure of these binding fragments, which may interfere in binding of cyc to NIK, can be designed. Such organic molecules can be used as drugs that can modulate NIK action and will be of value in preventing inflammatory response and other disorders in which NIK is involved in their pathogenesis.

A possible mechanism underlying modulation of NIK activity by cyc, may be enhanced phosphorylation of NIK upon cyc / NIK interaction. *In vitro* kinase assay showed a three-fold enhancement by cyc of NIK self- and IKK1-phosphorylation. Thus, the result obtained in the in vitro kinase assay supports the hypothesis that modulation of NIK activity by cyc may be enhanced phosphorylation of NIK upon cyc / NIK interaction.

NIK activation appears to have strict structural-requirements. Even though both aly-NIK and wild type NIK showed binding to cyc and similar levels of NF- κ B activation upon overexpression, cyc co-expression did not enhance NF- κ B activation by aly-NIK.

Induction of the LT β receptor by its ligand, results in NF- κ B activation. It is suggested in the literature that NIK is activated by inducing the LT β receptor with its ligand. The effect of overexpressing the intracellular domain of cyc polypeptide (cycICD) or its 41 membrane distal domain (41MDD) was tested when NF- κ B is activated by triggering the LT β receptor and this activation is believed to be mediated by endogenous NIK. ICDcyc expression enhanced the NF- κ B activation by LT β by 2.5 fold, while 41MDD expression inhibited by 50% NF- κ B activation by LT β . These results show that the cycICD polypeptide or the 41 MDD can modulate signaling triggered through the LT β receptor and demonstrating, once more, that ICD polypeptide or fragments thereof may serve as candidates for peptide based drug designing. Such drugs may modulate NIK action and therefore are valuable in preventing or alleviating diseases in which the action of NIK is involved in their pathogenesis. NIK has been shown to induce NF- κ B activation, thus the cyc or NIK fragments of the invention may be used to treat and/or prevent diseases in which NF- κ B is involved in their pathogenesis.

“Intrabodies” are specific antibodies that are transduced in a cell and are capable of inhibiting an undesired associated reaction such as NIK and cyc, wherein said antibody when expressed will bind in the cell to a target molecules and/or ligand involved in the undesired associated reaction, expressing the antibody and letting said antibody bind to said target receptor and/or ligand. The use of intrabodies is disclosed in WO9914353.

Such intrabodies can be prepared against 41DDM and 41DPM or NIK 640-720 and transduced in cells for inhibiting NIK and cyc interactions in a disease in which the activity of NIK is involved in its pathogenesis.

NF- κ B is a heterodimeric transcription factor that can activate a large number of genes that code, *inter alia*, for proinflammatory cytokines such as IL-1, IL-2, and TNF alpha or IL-6. NF- κ B is present in the cytosol of cells, complexed with its naturally occurring inhibitor I. κ B. The stimulation of cells, for example, by cytokines, leads to the phosphorylation and subsequent proteolytic degradation of I- κ B. This proteolytic degradation leads to the activation of NF- κ B, which subsequently migrates into the nucleus of the cell and there activates a large number of proinflammatory genes.

In disorders such as rheumatoid arthritis (in the case of inflammation), osteoarthritis, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis, NF- κ B is activated beyond the normal extent. The inhibition of NF- κ B is also of benefit in cancer therapy, since it is employed there for the reinforcement of the cytostatic therapy. The polypeptides of the invention may be produced, in eukaryotic or eukaryotic expression systems, intracellular, periplasmic or may be secreted into the medium. The produced cycICD or fragments thereof and NIK640-720 may be recovered in soluble or insoluble form (inclusion bodies).

A vector comprising cycICD or fragments thereof and NIK640-720 DNA may be used for expression of the polypeptides in prokaryotic or eukaryotic systems.

An expression vector encoding an effective signal peptide, preferably the human growth hormone signal peptide, fused to the cDNA of cycICD or fragments thereof and NIK640-720 may be used for eukaryotic expression and secretion.

The present invention provides cycICD, NIK640-720 peptides derived therefrom, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, or salt thereof for the manufacture of a medicament for the treatment of inflammatory diseases.

A therapeutic or research-associated use of these tools necessitates their introduction into cells of a living organism. For this purpose, it is desired to improve membrane permeability of peptides, proteins and oligonucleotides. Derivatization with lipophilic structures, may be used in creating peptides and proteins with enhanced membrane permeability. For instance, the sequence of a known membranotropic peptide as noted above may be added to the sequence of the peptide or protein. Further, the peptide or protein may be derivatized by partly lipophilic structures such as the above-noted hydrocarbon chains, which are substituted with at least one polar or charged group. For example, lauroyl derivatives of peptides have been described by Muranishi et al., 1991. Further modifications of peptides and proteins comprise the oxidation of methionine residues to thereby create sulfoxide groups, as described by Zacharia et al. 1991. Zacharia and co-workers also describe peptide or derivatives wherein the relatively hydrophobic peptide bond is replaced by its ketomethylene isoester (COCH₂). These and other modifications known to the person of skill in the art of protein and peptide chemistry enhance membrane permeability.

Another way of enhancing membrane permeability is the use receptors, such as virus receptors, on cell surfaces in order to induce cellular uptake of the peptide or protein. This mechanism is used frequently by viruses, which bind specifically to certain cell surface molecules. Upon binding, the cell takes the virus up into its interior. The cell surface molecule is called a virus receptor. For instance, the integrin molecules CAR and AdV have been described as virus receptors for Adenovirus, see Hemmi et al. 1998, and references therein. The CD4, GPR1, GPR15, and STRL33 molecules have been identified as receptors/co-receptors for HIV, see Edinger et al. 1998 and references therein.

Thus, conjugating peptides, proteins or oligonucleotides to molecules that are known to bind to cell surface receptors will enhance membrane permeability of said peptides, proteins or oligonucleotides. Examples for suitable groups for forming conjugates are sugars, vitamins, hormones, cytokines, transferrin, asialoglycoprotein, and the like

molecules. Low et al., USP 5,108,921, describes the use of these molecules for the purpose of enhancing membrane permeability of peptides, proteins and oligonucleotides, and the preparation of said conjugates.

Low and co-workers further teach that molecules such as folate or biotin may be used to target the conjugate to a multitude of cells in an organism, because of the abundant and unspecific expression of the receptors for these molecules.

The above use of cell surface proteins for enhancing membrane permeability of a peptide, protein or oligonucleotide of the invention may also be used in targeting said peptide, protein or oligonucleotide of the invention to certain cell types or tissues. For instance, if it is desired to target cancer cells, it is preferable to use a cell surface protein that is expressed more abundantly on the surface of those cells. Examples are the folate receptor, the mucin antigens MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC7, the glycoprotein antigens KSA, carcinoembryonic antigen, prostate-specific membrane antigen (PSMA), HER-2/neu, and human chorionic gonadotropin-beta. The above-noted Wang et al., 1998, teaches the use of folate to target cancer cells, and Zhang et al. 1998, teaches the relative abundance of each of the other antigens noted above in various types of cancer and in normal cells.

The protein, peptide or oligonucleotide of the invention may therefore, using the above-described conjugation techniques, be targeted to certain cell type as desired. For instance, if it is desired to inhibit activation of NIK in cells of the lymphocytic lineage, cycICD, fragment thereof, mutants and derivatives of the invention may be targeted at such cells, for instance, by using the MHC class II molecules that are expressed on these cells. This may be achieved by coupling an antibody, or the antigen-binding site thereof, directed against the constant region of said MHC class II molecule to the protein or peptide of the invention. Further, numerous cell surface receptors for various cytokines and other cell communication molecules have been described, and many of these molecules are expressed with in more or less tissue- or cell-type restricted fashion. Thus, when it is desired to target a subgroup of T cells, the CD4 T cell surface molecule may be used for

producing the conjugate of the invention. CD4-binding molecules are provided by the HIV virus, whose surface antigen gp42 is capable of specifically binding to the CD4 molecule.

The proteins, peptides and antisense sequences of the invention may be introduced into cells by the use of a viral vector. The use of vaccinia vector for this purpose is detailed in chapter 16 of Current Protocols in Molecular Biology. The use of adenovirus vectors has been described e.g. by Teoh et al., 1998, Narumi et al, 1998, Pederson et al, 1998, Guang-Lin et al., 1998, and references therein, Nishida et al., 1998, Schwarzenberger et al 1998, and Cao et al., 1998. Retroviral transfer of antisense sequences has been described by Daniel et al. 1998.

When using viruses as vectors, the viral surface proteins are generally used to target the virus. As many viruses, such as the above adenovirus, are rather unspecific in their cellular tropism, it may be desirable to impart further specificity by using a cell-type or tissue-specific promoter. Griscelli et al., 1998 teach the use of the ventricle-specific cardiac myosin light chain 2 promoter for heart-specific targeting of a gene whose transfer is mediated by adenovirus.

Alternatively, the viral vector may be engineered to express an additional protein on its surface, or the surface protein of the viral vector may be changed to incorporate a desired peptide sequence. The viral vector may thus be engineered to express one or more additional epitopes, which may be used to target, said viral vector. For instance, cytokine epitopes, MHC class II-binding peptides, or epitopes derived from homing molecules may be used to target the viral vector in accordance with the teaching of the invention.

The present invention encompasses pharmaceutical compositions comprising one or more active substance selected from one or more of cyc fragments such as ICDcyc, 41MDD, 44MPD and or the NIK fragment NIK640-720 and/or DNA or vectors harbouring their sequences.

The definition of "pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered. For example, for parenteral administration, the active protein(s) may be formulated in a unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

The active ingredients of the pharmaceutical composition according to the invention can be administered to an individual in a variety of ways. The routes of administration include intradermal, transdermal (e.g. in slow release formulations), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intracranial, epidural, topical, and intranasal routes. Any other therapeutically efficacious route of administration can be used, for example absorption through epithelial or endothelial tissues or by gene therapy wherein a DNA molecule encoding the active agent is administered to the patient (e.g. via a vector) which causes the active agent to be expressed and secreted in vivo. In addition, the protein(s) according to the invention can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, excipients, carriers, diluents and vehicles.

For parenteral (e.g. intravenous, subcutaneous, intramuscular) administration, the active protein(s) can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle (e.g. water, saline, dextrose solution) and additives that maintain isotonicity (e.g. mannitol) or chemical stability (e.g. preservatives and buffers). The formulation is sterilized by commonly used techniques.

The bioavailability of the active protein(s) according to the invention can also be ameliorated by using conjugation procedures which increase the half-life of the molecule in the human body, for example linking the molecule to polyethylenglycol, as described in the PCT Patent Application WO 92/13095.

The present invention relates to a method of enhancing or inhibiting NIK response in a patient in need, e.g. a patient suffering from inflammatory disease and/or cancer, comprising administration of a therapeutically effective amount of cycICD and/or NIK640-720 or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

A "therapeutically effective amount" is such that when administered, the IDC cyc and/or NIK640-720 and/or fragments thereof results in modulation of the biological activity of NIK. The dosage administered, as single or multiple doses, to an individual may vary depending upon a variety of factors, including the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled in the art, as well as in vitro and in vivo methods of determining the activity of IDC cyc and fragments thereof.

The cyc and fragments thereof such as 44MPD and 41MDD, and/or NIK640-720 can be used in an assay to screen for potential therapeutically valuable molecules which inhibit cyc binding to NIK. Cells expressing NIK cyc and optionally the above fragments and the reporter gene luciferase under an NF- κ B inducible promoter, can be exposed to a variety of individual synthetic organic compounds created by combinatorial chemistry and NF- κ B activation in treated cells versus control cells can be monitored and quantitated by the luciferase assay according to the invention. NIK may be activated by overexpressing using recombinant technics or by receptor-ligand induction. The compounds tested may be obtained not only through combinatorial chemistry, but also from other high throughput synthesis methods. Automated techniques enable the rapid synthesis of libraries of molecules, large collections of discrete compounds, which can be screened. Producing larger and more diverse compound libraries increases the likelihood of discovering a useful drug within the library. For high throughput screening robots can be used to test inhibition of recruitment or disruption of signalosome formation by thousands of compounds.

Alternatively, co-immunoprecipitation assay as such described in the examples below or immunoassays able to monitor binding of NIK and cyc, can be used for the detection of polypeptide or organic molecules inhibiting such binding. Such assay requires specific antibodies to the proteins and can be carried with intact NIK and cyc or with the fragments thereof such as NIK640-720, 41MDD, 44MPD and ICDcyc.

In addition screening for molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction comprising a polypeptide comprising the intracellular domain of the cyc or a mutein, fusion protein, functional derivative, active

fraction, circularly permuted derivative or fragment thereof comprising: coating or capturing (by a specific antibody bound to the plate) one of the proteins (e.g NIK or NIK640-720) in a plate and detecting the binding of the other protein (e.g. cyc, ICDcyc or fragments thereof) bound to the plate with specific antibody in the presence or absence of organic compounds.

The invention will be now illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Detection of proteins interacting with NIK by the two-hybrid system method:

The two-hybrid system method in yeast, widely used for detecting in-vivo protein-protein interaction, has been used to screen a DNA expression library to find and identify proteins that interact with NIK (see details in Example 8). A human bone marrow library has been selected based on evidence indicating a pivotal role of NIK in the lymphoid system development and function.

The N-terminal region of NIK contains a negative regulatory domain (NRD), which interacts with the C-terminal region of NIK, thereby inhibiting the binding of NIK to its substrates (IKK alfa and p100). Interaction between the C- and N terminal region of NIK prevents binding of NIK to its substrates. The C-terminal domain of NIK was found to be responsible for the binding of NIK to several key regulatory proteins such as TRAF-2, IKK-1 and to P100, suggesting that this domain may bind additional proteins, which are important for modulating its activities. Therefore, introducing the entire molecule, as bait in the two-hybrid system is undesirable since the C-terminal domain may be occluded by the NRD. Thus, the C-terminus of NIK (amino acids 624-947) has been used as the bait in a two-hybrid screen (for details see Example 8).

More than 5000 clones appeared on the selection plates. About half of the resistant clones were analyzed by α -gal assay and approximately 60% of them turned out positive with

varying intensity of blue colour. Plasmids were isolated and purified from 800 colonies. The DNA inserts of 400 plasmids out of the 800 (chosen according to colour intensity which is indicative of affinity of binding) were amplified, by polymerase chain reaction (PCR) using primers corresponding to the flanking sequences of the inserts in the cDNA library, and sequenced. Most of the preys detected, turned out to be non-specific e.g.: 80% of the DNA inserts corresponded to 3' and 5' untranslated regions of various genes and 10% to DNA inserts encoding immunoglobulins. The remaining 10% corresponded to segments encoding regions of proteins. Some of the positive colonies turned blue 4-8 days after seeded, some after about 8-12 days, and others became coloured late, up to 12-16 days after seeding. The speed of the colour development in positive colonies is indicative of the strength of protein-protein interaction i.e. the faster the colour appears, the stronger the interaction.

One of the binding proteins found, the IL-2 gamma chain receptor, was chosen for further analysis. The IL-2 gamma chain receptor is a subunit of the IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21 receptor complexes, therefore it is commonly dubbed as the 'common γ chain' (cyc).

Example 2

Evaluation of IL-2 gamma chain – NIK interactions in a mammalian environment:

The detection of a specific interaction between two different mammalian proteins in a two-hybrid system in yeast does not necessarily imply that there exists a corresponding interaction between the proteins in a native mammalian environment. Therefore, in order to verify NIK and cyc interaction in a mammalian environment, co-immunoprecipitation studies of NIK and cyc were carried out in lysates of 293-T cells overexpressing these proteins (for details see Example 9).

To overexpress NIK and cyc, 293-T cells were co-transfected with equal amounts of NIK and cyc expression plasmids (pcS3MTNIK, myc tag at its N-terminus and pcDNA3 cyc respectively, both plasmids having similar molecular weight). The overexpressed proteins were immunoprecipitated with antibodies specific for one of the proteins (e.g. NIK) and the presence of a coprecipitated protein (e.g. cyc) was detected by Western blots analysis.

Figure 2 summarized the results of Western blot analysis of immunoprecipitates detected with anti cyc antibodies. The samples analysed were the following: 1- a lysate of cells overexpressing cyc and immunoprecipitated with anti cyc. This sample is the positive control for the immunoprecipitation method. A strong signal corresponding to the molecular weight of cyc was observed. 2- a lysate of cells overexpressing cyc alone and immunoprecipitated with anti NIK antibody. This sample in the experiment was performed to check co-immunoprecipitation of cyc with endogenous NIK protein, which is present at minor concentrations and probably in inactive form, and also to check the specificity of the anti NIK antibodies. A protein with a molecular weight corresponding to cyc was not detected in the blot. 6- a lysate of cells overexpressing both myc tagged NIK and cyc and immunoprecipitated with anti myc antibody. Cyc is co immunoprecipitated together with NIK, demonstrating that cyc-NIK interaction occurs also in the native environment. 3- the same as 6 with the difference that NIK is exchanged for the NIK aly mutant (mutation in human is G860R corresponding to aly mutation in mouse G855R). Cyc is co-immunoprecipitated with NIK aly mutant indicating that the mutant is capable of binding cyc as efficient as the wild type NIK. 4- a lysate of cells overexpressing the C-terminus of NIK (amino acids 624-947), the same fragment of NIK employed as bait in the two-hybrid system. A band corresponding to cyc was not detected in the blot. 5- a lysate of cells overexpressing both the C-terminus of NIK and cyc and immunoprecipitation with anti NIK antibody. Cyc efficiently coprecipitated with the C-terminus of NIK.

These results show that cyc is efficiently co-precipitated with either the C-terminus of NIK (used as the bait in the two hybrid system in which the cyc was identified) or with the full length NIK.

Figure 3 summarizes the results of Western blot analysis of immunoprecipitates and total cell lysates detected with anti NIK antibody. The samples analysed were the following: 3- a lysate of cells overexpressing NIK alone immunoprecipitated with anti NIK. This sample is the positive control for the immunoprecipitation method. A strong signal of the molecular weight corresponding to NIK was observed. 2- a lysate of cells overexpressing NIK alone and immunoprecipitated with anti cyc antibody. A protein with a molecular weight corresponding

to NIK could not be detected. This result demonstrates also the specificity of the cyc antibodies.

1- lysates of cells overexpressing both NIK and cyc immunoprecipitated with anti cyc antibody. The results show that NIK is effectively coimmunoprecipitated with cyc. 5- is a lysate of non-transfected cells, 4 and 6 are lysates of cells overexpressing both NIK and cyc or cells overexpressing NIK alone respectively before immunoprecipitation. A strong band corresponding to the molecular weight of NIK was observed in the blot, demonstrating that it is overexpressed.

The results obtained by Western blots analysis of immunoprecipitates showed bi-directional precipitation of NIK and cyc demonstrating that their interaction occurs also in mammalian cells. The C-terminal domain of NIK, the full length NIK and the mutant NIK^{Ala} (NIK-G860R) are all coimmunoprecipitable by cyc.

Example 3

Mapping the region in cyc responsible for binding NIK:

To define the domain of cyc responsible for binding NIK, deletion mutants of cyc have been created and their binding to NIK analysed (Figure 1).

The deletion mutants were created by sequentially introduction of stop codons in the cytoplasmic domain of cyc, in gaps of 10-20 amino acids. The DNA encoding the full-length cyc or its deletion mutants were introduced into the pGADT7 prey vector (Clontech) for testing their binding to NIK in the SFY526 heterologous yeast strain by the two hybrid assay. The SFY526 yeast strain is prototrophic for TRP and Leu. pGBKT plasmids (bait vector) have the Trp1 wild type gene and pGAD has the wild type Leu2 gene. Thus, only doubly transfected yeast will grow on selective Leu Trp media. Functional GAL4 will be restored in doubly transfected yeast when the chimeric proteins fused to GAL4 domains interact, bringing the activation domain and DNA binding domain of GAL4 to close proximity. The level of LAC-Z expression is indicative of the strength of the protein-protein interaction. Lac-Z activity was assessed by the standard beta-gal/colony lift filter assay (Clontech, Yeast Protocol Handbook, Chapter VI).

Since introduction of *cyc* and mutants into the pGADT7 prey vector for assessing their binding to NIK as bait manifested high non-specificity, the interactions were tested in the reverse orientation: i.e. deletion mutants were cloned into the bait vector and NIK or C-terminus of NIK (residues 624-947) in the prey vector. The results summarized in Table 1 show that none of the deletions, but the cytoplasmid domain of *cyc* (ICD) alone showed strong binding, to both NIK and NIK C-terminus. The binding of most of the ICD (lacking 5 amino acid from its proximal membrane domain) to both NIK and C-terminus NIK was stronger than that of the full-length *cyc* molecule. A 50% reduction in affinity to NIK was observed by deleting 12 amino acids or 44 amino acids at the membrane distal end of cycICD.

Table 1.

| cyc amino acid residues | NIK624-947 (C-terminal domain) | NIK | Lamin |
|---|-----------------------------------|-----|-------|
| Full length (1-369) | +- | - | - |
| 1-357 | - | - | * |
| 1-325 | - | - | * |
| 1-303 | - | - | * |
| 1-282 | - | - | * |
| 289-369 (most of ICD) | ++++ | +++ | - |
| 289-357 (12 aa deleted from the membrane distal domain) | ++ | * | * |
| 289-325 (44 aa deleted from the ICD) | ++ | * | * |

* Not tested

The results obtained with the different deletion mutants indicate that the membrane distal domain of *cyc* is involved in binding to NIK. Thus binding of a 41 amino acid polypeptide from the membrane distal domain of *cyc*, corresponding to residues 329-369 (dubbed 41 MDD) was analysed.

Table 2.

| Bait | Prey | | |
|--|---|--------------------------------|-----|
| | <i>cyc</i> 329-369 (41 MDD polypeptide) | NIK624-947 (NIK C-terminus) | NIK |
| NIK624-947 (C-terminus) | +++ | * | * |
| NIK | + | * | * |
| <i>cyc</i> 329-369 (41 MDD polypeptide) | * | +/- | +/- |
| Lamin | - | - | - |

The binding of 41 MDD polypeptide to full length NIK or C-terminus NIK was tested in both orientations (i.e. 41 MDD as the pray and NIK as the bait and vice versa). The results obtained are shown in Table 2. The interaction is relatively weak when NIK serves as the prey partner, but strong when NIK serves as the bait. The interaction of the 41 MDD is stronger with the C-terminus of NIK than with the full length NIK. These results confirmed that the 41 MDD polypeptide is involved in binding to NIK.

Similarly, two hybrid experiments were carried out with constructs comprising intracellular fragments located close to the membrane proximal domain (MPD) of cyc. The results suggested that a region of 44 amino acids, spanning amino acid residues 282-235 (44MPD) can also bind NIK.

Co-immunoprecipitation studies confirmed this results. The 44MPD fused to GST and mycNIK were overexpressed in cells, the cells lysed and immunoprecipitated with anti GST (see Example 9). The immunoprecipitates were analysed by western blots. The bound NIK was detected with antimyc antibodies. The results demonstrated that the 44MPD fragment also binds NIK(not shown).

Mutagenesis studies were carried out in ICDcyc, in residues located at the 41 MDD, in order to define specific amino acids interacting with NIK. The interaction of proline rich motifs in signaling proteins with their cognate domains is well documented (Kay BK, Williamson MP, Sudol M. FASEB J 2000 Feb 14 (2): 231-421). 20% of the amino acids in the 41 MDD amino acids are prolines. Therefore, two consecutive prolines were mutated to alanines at two different sites within the 41 MDD were mutated: 1- PP 336,337AA and 2- PP360, 361AA.

The mutation were carried out employing polymerase chain reaction (PCR) using the following primers:

For the generation of the PP336, 337AA mutants the following primers were used:

5' ctcgtcagtgagattgccgcaaaaggaggggcccttg (SEQ ID NO: 7)

5' caaggcccccctcctttgcggcaatctcactgacgag (SEQ ID NO: 8)

For the generation of the PP360, 361AA mutants the following primers were used:

5' gcccctactgggcccgcatgttacaccctaaag (SEQ ID NO: 9)

5'ctttagggtaacatgcggcccccagtagggc (SEQ ID NO: 10)

In addition mutations were carried out in the 41 MDD in residues different from proline e.g. K338, E344 and W358.

For the generation of the K338A mutant the following primers were used:

5' gtcagtgagattcccccagcaggagggccctgggag (SEQ ID NO: 11)

5' ctccccaaaggccccctcgctggggaaatctcactgac (SEQ ID NO: 12)

For the generation of the E344A mutant the following primers were used:

5' ggagggcccttggggcggggcctgggcctcc (SEQ ID NO: 13)

5' ggaggccccaggccccggcccaaggccccctcc (SEQ ID NO: 14)

For the generation of the W358A mutant the following primers were used:

5' cagcatagccccctacgcggcccccattgtac (SEQ ID NO: 15)

5' gtaacatggggggccgcgtagggctatgctg ((SEQ ID NO: 16)

The mutated version of cyclCD were used as the bait and its interaction with NIK-C terminus was tested in the two hybrid system as described in Example 8.

Table 3.

| Bait | Prey-NIK624-947 (C-terminus) |
|----------------------------|------------------------------|
| cyc 289-369 (ICD) | ++++ |
| cyc289-369 (PP 336, 337AA) | +++ |
| cyc289-369 (PP 360, 361AA) | ++ |
| cyc289-369 K338A | +++ |
| cyc289-369 E344A | +++ |
| cyc289-369W358A | +++ |
| Lamin | - |
| TRAF2 | + |

The results are summarized in Table 3. The replacement of prolines for alanine in residues 360 and 361 reduced the affinity to NIK by 50 %, in contrast to other replacements, which failed to show substantial effect.

The results obtained of cyc mutagenesis demonstrate that the prolines at residues 360 and 361, which are located within the 41MDD region, are important for the binding to NIK.

Example 4

Effect of cyc and its deletion mutants on the NF-κB induction mediated by NIK overexpression:

One experimental way to induce NF-κB activation in cells is by overexpressing NIK.

To check the effect of cyc on NF- κ B activation mediated by NIK, cells were transiently transfected with the reporter plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3 luciferase) and expression plasmids encoding NIK alone (pcS3MTNIK) or together with an expression plasmid encoding cyc (pcDNA3cyc). Activation of NF- κ B was monitored by the luciferase reporter assay (for details see Example 10).

293-T cells were transfected with pcS3MTNIK and pcDNA3 luciferase. NF- κ B activation was measured indirectly by measuring the luciferase activity present in the cells. To assess the effect of cyc on NIK mediated NF- κ B activation pcDNA3cyc was co-transfected with pcS3MTNIK and pcDNA3 luciferase. Several cotransfections were carried out to test the effect of different concentrations of pcDNA3cyc with a constant concentration of pcS3MTNIK and pcDNA3 luciferase. 24 hours post transfection the cells were harvested, lysed and luciferase activity monitored.

The results of this experiment are summarized in Figure 4. NIK overexpression alone induces expression of luciferase activity indicating that NF- κ B is activated. This increase in luciferase activity was not observed in cells transfected with either the empty plasmid alone (pc) or the reporter gene and empty plasmid (pc+luc). The effect of cyc on NF- κ B activation was found to depend on its concentration relative to NIK e.g. if cyc was expressed at lower concentrations than NIK, it potentiated NIK's effect (NIK 1 μ g and cyc 0.1 μ g plasmid DNA), while if present at equal or higher concentration, it inhibited NIK's effect (NIK 1 μ g and cyc 1 μ g plasmid DNA). Transfection of the cyc plasmid alone did not result in NF- κ B activation (Fig 5).

The C-terminus of NIK (residues 624-947) can be regarded as a dominant negative mutant (dnNIK) since it can bind to substrates and cyc (see Example 2), but is catalytically inactive. The effect of dnNIK overexpression on the enhancement in NF- κ B activation observed in cells expressing low concentration of cyc and overexpressing NIK was monitored. The results are summarized in Figure 5. As previously shown overexpression of NIK alone induced

activation of NF- κ B as evidenced by the increase in luciferase activity. Overexpression of dnNIK together with NIK inhibited this NF- κ B activation. A further enhancement in NF- κ B activation mediated by NIK was observed when cyc was expressed at low concentration. However, this enhancement of NF- κ B activation was blocked by overexpression of dnNIK. This result confirms that the NF- κ B inductive effect of cyc is exerted via NIK.

The human AlyNIK mutant (mutation in human is G860R corresponding to aly mutation in mouse G855R) was shown to bind cyc by the two-hybrid method (Example 2). Overexpression of this mutant alone induced NF- κ B activation as efficient as the wild type NIK (Figure 6). The effect of cyc on NF- κ B activation mediated by aly NIK mutant was tested and is summarized in Figure 6. Expression of cyc did not enhance NF- κ B induction mediated by alyNIK. Thus, despite that alyNIK mutant is capable of binding cyc, its activity inducing NF- κ B is not affected by cyc.

As shown above, the effect of full length cyc on NF- κ B activation mediated by NIK overexpression is concentration dependent, e.g. to inhibit NIK mediated NF- κ B activation a high or equal concentrations of cyc relative to NIK is required. In contrast, to enhance NIK mediated NF- κ B activation, a low concentration of cyc relative to NIK is required.

The effect of overexpression of the 41 MDD (41 residues in the distal membrane domain of cyc, shown to bind NIK) on NIK induced and cyc enhanced NF- κ B activation was tested.

Expression of intracellular cyc and fragments thereof in mammalian cells by transfection fails to give an appreciable amount of protein as evidenced by Western blot analysis (not shown). This may be due to the instability imposed by deletion of the transmembrane domain and extracellular domain. The intracellular domain contains a PEST domain, which might be exposed in the cycICD and fragments thereof and prone to proteases present in the cells. To solve this problem GST fusion of 41 MDD was generated to stabilize it and the effect of 41MDD-GST fusion protein on NF- κ B activation induced by NIK and cyc was tested.

150000 293-T cells were seeded per well in 6 well plates. 24 hours later the cells were transfected with a total DNA concentration at 3 μ g/well (carrier DNA pcDNA). pcDNAcyc was used at a concentration of 50 ng/well to induce enhancement of NF- κ B activity mediated by NIK. PgGST and a plasmid encoding the fusion protein GST-41MDD were used at high concentration 2 μ g/well, pcS3MTNIK and pcDNA3luciferase at 0.5 μ g/well. 24 hours post transfection, cells were harvested in 100 μ l extraction buffer and lysed by repeated freezing and thawing. Lysates were precleared by centrifugation (14000 rpm, microfuge 1 min.). Luciferase activity of 10 μ l of the lysate was assayed in 360 μ l of assay buffer. The results are summarized in Figure 7. NF- κ B induction is enhanced by overexpression of NIK and low expression of cyc. However, in a sample where the fusion protein GST-41 MDD is coexpressed with NIK and cyc, the activation levels of NF- κ B are below the levels observed following overexpression of NIK alone. This result indicates that the 41 MDD, similar to the whole cyc, inhibits NIK dependent NF- κ B activation when present at higher concentrations relative to NIK.

The effect of cyc and various mutants deleted at the C-terminal end of cyc (Figure 1) on NF- κ B activation induced by NIK was tested. The concentration of plasmid encoding the cyc and cyc mutants used was 0.5 μ g/ml the same concentration as the plasmid encoding NIK. Under these conditions, the full-length cyc is expected to cause inhibition of NF- κ B activation mediated by NIK.

150000 Hela cells were seeded per well in 6 well plates. 24 hours later transfection was performed keeping the total DNA constant at 2 μ g/well (pcDNA3 was used as carrier DNA). Plasmids encoding full-length cyc and all its deletion mutants were used at a concentration of 0.5 μ g/well. NIK and luciferase encoding plasmids were also used at 0.5 μ g/well. 24 hours post transfection, cells were harvested in 100 μ l extraction buffer and lysed by repeated freezing and thawing. Lysates were precleared by centrifugation (14,000 rpm in a microfuge, 1 min.). Luciferase activities of the lysates were assayed in 360 μ l of assay buffer.

The results are summarized in Fig. 8. Full-length cyc expressed at the same concentration as NIK inhibits NF- κ B activity. Expressing cyc having progressive deletions in the membrane distal domain (Figure 1), a domain that was shown to participate in binding to NIK, resulted

in a concomitant decrease in inhibition of NF- κ B activity. Deletion mutants with stop codons at residues 325 and 303 did not affect the activity of NIK.

These results confirm that the residues present in the membrane distal domain of cyc (from residues 325 to 369) participate in the binding of NIK and are important for modulating its activity.

Example 5

Effect cyc on the kinase activity of NIK:

In the previous examples it has been shown that cyc binds to NIK and modulates its activity. A possible mechanism underlying this regulation may be enhanced phosphorylation of NIK occurring upon cyc / NIK interaction.

To test the above hypothesis, NIK phosphorylation was assayed in-vitro in sample of cells overexpressing cyc alone (Fig 9 lane 1), NIK alone (Fig 9 lane 3 from the left), or NIK together with cyc (Fig 9 lane 2 from the left), or NIK together with the kinase IKK (Fig 9 lane 4 from the left) lysed and immunoprecipitated with anti NIK antibodies (For details see Example 11).

Kinase reaction was carried out with 5 μ ci γ 32 P-ATP as previously described (Uhlik et al. 1998). The results in Figure 9 show that cyc alone did not display any kinase activity (Fig 9 lane 1 from the left). A three fold increase in phosphorylation of NIK self- and IKK1-phosphorylation was observed in the presence of cyc (compare lines 2 and 3). This result indicates that cyc may modulate the activity of NIK by inducing its phosphorylation.

Example 6

Effect of cyc in modulating signal transduced through the LT β receptor:

Induction of the LT β receptor by its ligand, results in NF- κ B activation. It is suggested in the literature that NIK participates in signaling through the LT β receptor. Thus, the effect of

overexpressing the whole cytoplasmic cyc polypeptide or the 41 distal domain (329-369) on NF- κ B activation mediated by the LT β receptor was tested. Activation of NF- κ B was monitored by the luciferase reporter assay (for details see Example 10).

A cell line was prepared from mouse embryonic fibroblast cells, which are generally known to express the LT β receptor. 10^5 cells of the above line were seeded per well in 6 well plates. 24 hours later transfection was performed (with Gene porter transfection reagent, Gene therapy systems) with the plasmid pcGST ICcgc expressing the intracellular domain of cyc (cyc IDC) fused to GST or with pcGST41MDD expressing the 41 distal domain of cyc fused to GST and the expression plasmid encoding luciferase reporter protein under the control of an NF- κ B inducible promoter (pcDNA3 luciferase). NF- κ B activation was measured indirectly by measuring the luciferase activity present in the cells.

Total DNA concentration was normalized to 2 μ g/well with empty vector (pcDNA3). pcGST ICcgc and pcGST41MDD were used at a concentration of about 1 μ g/well. 24 hours after the transfection, cells were stimulated with 50ng/ml recombinant LT β (cat# L-5162, Sigma) for 1 hour.

The results are summarized in Figure 10. Expression of the intracellular domain of cyc, enhanced the NF- κ B activation by LT β by 2.5 fold, while the expression of the membrane distal 41 amino acids inhibited by 50% NF- κ B activation by LT β .

The above results suggest that cyc may be involved in signaling through the LT β receptor. The cyc 41 distal domain inhibits signaling through LT β receptor, indicating that this polypeptide or fragments thereof may serve as candidates for peptide based drug designing. Such drugs may modulate NIK action and therefore are valuable in preventing or alleviating inflammatory responses or in modulatory immunoregulatory processes.

Example 7

Mapping the region in NIK involved in the interaction with cyc:

The binding region in NIK was determined by testing the interaction of a series of NIK deletion mutants with cyc employing the yeast two-hybrid system. The truncated mutants of

NIK were cloned into the pGBT9 two-hybrid bait vector and cyc was cloned into the pGADT7 prey vector. The binding was tested in the SFY526 heterologous yeast strain, by beta-gal assay.

| Bait | Strength of interaction with Prey | | |
|------------|-----------------------------------|-------|-------|
| | cyc | Traf2 | Lamin |
| NIK624-947 | ++++ | +++ | - |
| NIK | -/+ | + | - |
| NIK 1-367 | - | * | * |
| NIK 1-769 | - | * | * |
| NIK 1-820 | ++ | * | * |
| Lamin | - | - | * |

* Not tested

The results show that the cyc binding region in NIK resides in 196 amino acids at the C-terminus (residues 624-820).

To define more precisely the domain of NIK responsible for binding cyc, more deletion mutants of NIK were created and their binding to cyc was analysed by coimmunoprecipitation. 293T cells were transfected with vector encoding cyc and His tagged NIK deletion mutants and the binding of the different deletion mutants to cyc was tested by coimmunoprecipitation (see details in Example 9). Antibody against the cyc was used for immunoprecipitation and anti His antibodies were used to detect His-NIK deletion mutants of the immunoprecipitated material on Western blots. The results are summarized in Table 4.

Table 4.

| Construct | Binding to cyc |
|----------------------------|----------------|
| NIK 1-947 (full length) | + |
| NIK 1-821 | + |
| NIK 1-771 | + |
| NIK 1-720 | + |
| NIK 1-640 | - |

The results indicate that a domain of NIK comprising 81 amino acid residues, located between amino acids 640 and 720 (SEQ ID NO: 18), is responsible for binding cyc.

Example 8

The two hybrid system method:

The two-Hybrid system used for screening was the Matchmaker version III (Clontech). In this system the bait gene (NIK gene) is expressed as a fusion to the GAL4 DNA binding domain (DNA-BD), while the prey genes or cDNA library is expressed as a fusion to the GAL4 activation domain (AD). When the DNA-BD and AD are brought into proximity, transcription of four reporter genes is activated (encoding HIS, ADE, lacZ and α -gal).

A human bone marrow library (Clontech cat# HY4053AH) has been selected as the prey, based on evidences indicating a pivotal role of NIK in the lymphoid system development and function.

Clones growing on plates under high stringency conditions i.e. in plates without LEU (selection marker for the bait encoding plasmid), TRP (selection marker for the prey encoding plasmid), HIS and ADE and impregnated with substrates for detection of α -gal expression.

Plasmids were purified from positive clones by lysis of the yeast cells (with detergent and mechanical stress) followed by phenol extraction and ethanol precipitation of the DNA. cDNA inserts in the plasmids were amplified by PCR with flanking primers specific for the library vector pACT2. Individual amplified cDNAs were directly cloned into a mammalian expression vector for further biochemical analysis.

Example 9

Immunoprecipitation method:

For transfection 1.5 million 293-T cells were seeded into 10 cm plates. 24 hours later, calcium phosphate assisted cotransfection (Molecular Cloning 2nd edition 15.33) was carried out with myc tagged NIK and cyc expression plasmids, maintaining a total DNA concentration of 20 μ g per plate. 30 hours later, cells were harvested and lysed in 1% NP-40 lysis buffer (0.5% NP-40, 10 mM Tris (PH 7.5), 150 nM NaCl, 1mM EDTA). Immunoprecipitations were carried out by incubating 16hours with the respective antibodies (rabbit polyclonal from Santa Cruz) directed either against the C-termini of cyc or directed against NIK which were pre-adsorbed to protein A sparse (rabbit polyclonal) or protein G sparse (mouse monoclonal). Immunoprecipitates were washed three times with lysis buffer and once with buffered saline. Beads were boiled in 40 μ l of Laemmli sample buffer and 20 μ l loaded in 10% SDS/PAGE. Proteins were blotted from the gel to a PVDF membrane and probed with anti cyc and anti NIK, followed by Goat anti rabbit antibody conjugated with horseradish peroxidase. Blots were developed by Enhanced Chemi Luminiscence (ECL) using Luminol (cat A8511, Sigma) as substrate.

Example 10

NIK mediated NF- κ B activation assay:

293-T cells (1.5×10^5 per well in 6-well plate) were transfected with the total DNA amount of 3 μ g per well. When needed the empty vector pCDNA was used as carrier DNA.

Cotransfections were carried out as described in Example 9 with 1 μ g of pcS3MTNIK and 0.5 μ g of pcDNA3 vector expressing luciferase under the control of HIV-LTR (Human immunodeficiency virus long terminal repeats), a promoter upregulated by NF- κ B. DNA encoding the cyc (pcDNA cyc) was introduced into pcDNA and used at 1/10, 1/2, and 1/1 ratio of the NIK expression vector concentration (the vectors have about the same molecular weight) 24 hours post transfection, cells were harvested in 100 μ l extraction buffer (0.1 M potassium phosphate, pH 7.8, 1 mM DTT) and lysed by repeating freezing and thawing (Liquid nitrogen and 1min. at 22°C). Lysates were pre-cleared by centrifugation (14,000 rpm, microfuge, 1 min.). Luciferase activity of 5 μ l lysate was assayed in 360 μ l buffer (20 mM potassium phosphate, 20 mM Glycyl-Glycine, 8.5 μ M Magnesium sulphate, 2 mM EGTA, 1 mM DTT, 1 mM ATP and 5 μ M D-luciferin (cat L-6882, Sigma).

Example 11

Kinase assay:

293-T cells (2×10^6 per 10cm plate) were transfected by the calcium phosphate method with 10 μ g of pcS3MTNIK and 10 μ g of pcDNAcyc or histidine tagged IKK1 (pcHISIKK1) maintaining total DNA concentration of 20 μ g per plate using empty pcDNA as the carrier DNA. 24 hours later, cells were harvested and lysed in 1 % NP-40 lysis buffer and immunoprecipitation carried out for 8 hours with rabbit anti NIK antibody pre-adsorbed to protein A sepharose beads. Kinase reaction was carried out with 5 μ ci γ^{32} P-ATP as previously described (Uhlik et al. 1998).

Example 12

Preparation and screen of non peptide small molecules inhibiting NIK- cyc interaction:

A library of small non peptide molecules are prepared by combinatorial chemistry. The design of combinatorial chemistry technology is well known in the art and is described e.g by Hermkens et al. (1996). Cells expressing NIK, cyc and the reporter plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3 luciferase) are exposed to individual synthetic organic compounds and NF- κ B activation is tested as described in example 4.

Compounds able to inhibit NF- κ B activation are selected for future testing.

Alternatively, cells are transiently transfected with cyc and the reporter plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3 luciferase) and exposed to individual synthetic organic compounds. Following exposure to synthetic compounds, NF- κ B activation is tested as described in example 6 when endogenous NIK is activated by ligand bindig to the corresponding receptor.

Compounds able to inhibit NF- κ B activation are selected for future testing.

Example 13

Interaction of endogenous NIK and cyc.

NIK and cyc interaction was demonstrated in a mammalian cell environment, in lysates of 293-T cells overexpressing these proteins (see Example 4). The following experiment was carried with endogenous proteins, in cells naturally expressing these proteins. Thus, peripheral blood mononuclear cells (PMBc) (500×10^6 cells) were incubated with IL-2, lysed and immunoprecipitated with anti cyc antibodies (for immunoprecipitation

see Example 9). Co-immunoprecipitated proteins bound to cyc were detected in Western blots using relevant antibodies. The candidate proteins tested for co-immunoprecipitation with cyc were those proteins normally present in the signalosome, such as NIK, IKK α (IKK-1), IKK β (IKK2), IKK γ (NEMO). The co-immunoprecipitated proteins were tested in lysates of cells tested at time-0 and after four-hour incubation with IL-2. The results summarized in Figure 16 A show that NIK is coprecipitated with cyc before and after stimulation with IL-2. Therefore NIK was found constitutively associated with cyc. Traces of IKK-1 was found in the basal level and upon 4 hours incubation with IL-2, other signalosome components i.e. IKK-2 and NEMO were recruited to the IL-2 receptor through the cyc. The results indicate that the IL-2 receptor common gamma chain is bound to NIK at a different location than the IKK-1 binding region.

To check whether the signalosome co-immunoprecipitated with cyc are active, the above immunoprecipitates were tested in a kinase assay (see details in Example 11) which monitors phosphorylation of GST-IKB α 1-54. The results summarized in Figure 16 B indicated that only immunoprecipitates from cells stimulated with IL-2 are capable of phosphorylating GS-IKB α 1-54.

Thus these results demonstrate that under physiological conditions, NIK is constitutively associated to cyc, and that this interaction is involved in IL-2 signalling and NIK dependent NFkB activation. Therefore inhibiting of cyc and NIK interaction will result in the inhibition IL-2 signalling activities and inhibition of NIK induced NFkB activation.

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CLAIMS:

1. The use of a polypeptide comprising the intracellular domain of the IL-2 receptor common gamma chain (cyc) or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof in the manufacture of a medicament for treatment of a disease in which NIK activation is involved in its pathogenesis.
2. The use according to claim 1, wherein the fragment is 41MDD.
3. The use according to claim 1, wherein the fragment is 44MPD.
4. The use according to anyone of claims 1 to 3, for the treatment of cancer.
5. The use according to anyone of claims 1 to 3, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
6. The use of an expression vector comprising a sequence encoding a polypeptide comprising the intracellular domain of the IL-2 common gamma chain, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment of a disease in which NIK activation is involved in its pathogenesis.
7. The use according to claim 6, wherein the fragment is 41MDD.
8. The use according to claim 6, wherein the fragment is 44MPD.
9. The use according to anyone of claims 6 to 8, for the treatment of cancer.
10. The use according to anyone of claims 6 to 8, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
11. The use of a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment of a disease in which NF- κ B activation is involved in its pathogenesis.

12. The use according to claim 11, wherein the fragment is 41MDD.
13. The use according to claim 11, wherein the fragment is 44MPD.
14. The use according to anyone of claims 11 to 13, for the treatment of cancer.
15. The use according to anyone of claims 11 to 13, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
16. The use of an expression vector comprising the coding sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment of a disease in which NF- κ B activation is involved in its pathogenesis.
17. The use according to claim 16, wherein the fragment is 41MDD.
18. The use according to claim 16, wherein the fragment is 44MPD.
19. The use according to anyone of claims 16 to 18, for the treatment of cancer.
20. The use according to anyone of claims 16 to 18, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
21. The use of a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, active fraction, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment and/or prevention of a disease resulting from excessive immune responses.
22. The use according to claim 21, wherein the fragment is 41MDD.
23. The use according to claim 21, wherein the fragment is 44MPD.
24. The use according to anyone of claims 21 to 23, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.

25. The use of an expression vector comprising a sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment and/or prevention of a disease resulting from excessive immune responses.
26. The use according to claim 25, wherein the fragment is 41MDD.
27. The use according to claim 25, wherein the fragment is 44MPD.
28. The use according to anyone of claims 25 to 27, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
29. The use of a polypeptide comprising an intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease in which NIK activation is involved in its pathogenesis.
30. The use according to claim 29, wherein the fragment is 41MDD.
31. The use according to claim 29, wherein the fragment is 44MPD.
32. The use according to anyone of claims 29 to 31, for the treatment of cancer.
33. The use according to anyone of claims 29 to 31, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
34. The use of a vector comprising the sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease in which NIK activation is involved in its pathogenesis.
35. The use according to claim 34, wherein the fragment is 41MDD.
36. The use according to claim 34, wherein the fragment is 44MPD.
37. The use of a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment

thereof, as a target in disease in which NF- κ B activation is involved in its pathogenesis.

38. The use according to claim 37, wherein the fragment is 41MDD.
39. The use according to claim 37, wherein the fragment is 44MPD.
40. The use according to anyone of claims 37 to 39, for the treatment of cancer.
41. The use according to anyone of claims 37 to 39, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
42. The use of an expression vector comprising the sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease in which NF- κ B activation is involved in its pathogenesis.
43. The use according to claim 42, wherein the fragment is 41MDD.
44. The use according to claim 42, wherein the fragment is 44MPD.
45. The use according to anyone of claims 42 to 44, for the treatment of cancer.
46. The use according to anyone of claims 42 to 44, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
47. A method of treatment and/or prevention of a disease in which NIK activation is involved in its pathogenesis, comprising administering to a host in need thereof an effective amount of a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
48. A method according to claim 47, wherein the fragment is 41MDD.
49. A method according to claim 47, wherein the fragment is 44MPD.
50. A method according to anyone of claims 47 to 49, for the treatment of cancer.

51. A method according to anyone of claims 47 to 49, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
52. A method of treatment and/or prevention of a disease in which NIK activation is involved in its pathogenesis, comprising administering to a host in need thereof an effective amount of a vector comprising a sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
53. A method according to claim 52, wherein the fragment is 41MDD.
54. A method according to claim 52, wherein the fragment is 44MPD.
55. A method according to anyone of claims 52 to 54, for the treatment of cancer.
56. A method according to anyone of claims 52 to 54, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
57. A method of treatment and/or prevention of a disease in which NF-κB activation is involved in its pathogenesis, comprising administering to a host in need thereof an effective amount of a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
58. A method according to claim 57, wherein the fragment is 41MDD.
59. A method according to claim 57, wherein the fragment is 44MPD.
60. A method according to anyone of claims 57 to 59, for the treatment of cancer.
61. A method according to anyone of claims 57 to 59, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
62. A method of treatment and/or prevention of a disease in which NF-κB activation is involved in its pathogenesis, comprising administering to a host in need thereof an effective amount of a vector comprising a sequence encoding a polypeptide

comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

63. The method according to claim 62, wherein the fragment is 41MDD.
64. The method according to claim 62, wherein the fragment is 44MPD.
65. A method according to anyone of claims 62 to 64, for the treatment of cancer.
66. A method according to anyone of claims 62 to 64, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
67. A method of treatment and/or prevention of a disease resulting from excessive immune responses, comprising administering to a host in need thereof an effective amount a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
68. A method according to claim 67, wherein the fragment is 41MDD.
69. A method according to claim 67, wherein the fragment is 44MPD.
70. A method according to anyone of claims 67 to 69, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
71. A method of treatment and/or prevention of a disease resulting from excessive immune responses, comprising administering to a host in need thereof an effective amount a vector comprising a sequence encoding a polypeptide comprising the intracellular domain of the cyc, or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
72. A method according to claim 71, wherein the fragment is 41MDD.
73. A method according to claim 71, wherein the fragment is 44MPD.
74. A method according to anyone of claims 71 to 73, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.

75. A method of screening for molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction comprising:

exposing cells expressing NIK, a reporter plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3 luciferase) and cyc or fragments thereof, to individual synthetic organic compounds, testing luciferase expression and isolating compounds capable of inhibiting or enhancing NF- κ B activity.

76. A molecule isolated according to claim 75.

77. A method of screening for organic molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction comprising co-immunoprecipitation of NIK and intracellular IL-2 receptor common gama chain or fragments thereof in the presence of individual organic molecules and detection of an organic molecule capable of inhibiting co-immunoprecipitation of NIK and cyc.

78. A molecule isolated according to claim 77.

79. A method of screening for organic molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction comprising: coating or capturing a first protein IL-2 receptor common gama chain or NIK to a plate and detecting the binding of a second protein NIK or IL-2 receptor common gama chain respectively in the presence of individual organic molecules and identificating of an organic molecule capable of inhibiting binding of the second protein to the coated plate.

80. A method according to claim 79, wherein the first protein is NIK or fragments thereof.

81. A method according to claim 79, wherein the first protein is cyc or fragments thereof.

82. A method according to anyone of claims 79 to 81, wherein the NIK fragment is NIK640-720.

83. A molecule isolated according to anyone of claims 79 to 82.

84. The use of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted

derivative or fragment thereof, in the manufacture of a medicament for treatment of a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.

85. The use of a vector comprising the coding sequence of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment of a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.
86. The use of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment and/or prevention of a disease resulting from excessive immune responses.
87. The use according to claim 86, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
88. The use of an expression vector comprising the coding sequence of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, in the manufacture of a medicament for the treatment and/or prevention of a disease resulting from excessive immune responses.
89. The use according to claim 88, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
90. The use of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.

91. The use of a vector comprising the coding sequence of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.
92. The use of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.
93. The use of an expression vector comprising the coding sequence of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof, as a target in a disease, wherein induction by a cytokine having the common gamma chain in its receptor is involved in the pathogenesis of the disease.
94. A method of treatment and/or prevention of a disease in which activation of a cytokine, having the common gamma chain in its receptor, is involved in the pathogenesis of the disease, comprising administering to a host in need thereof an effective amount of a polypeptide comprising a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
95. A method of treatment and/or prevention of a disease in which activation of a cytokine, having the common gamma chain in its receptor, is involved in the pathogenesis of the disease, comprising administering to a host in need thereof an effective amount of a vector encoding a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
96. A method of treatment and/or prevention of a disease in which NF- κ B activation is involved, comprising administering to a host in need thereof an effective amount of a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a

mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

97. A method according to claim 96, for the treatment of cancer.
98. A method according to claim 96, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
99. A method of treatment and/or prevention of a disease in which NF- κ B activation is involved in its pathogenesis, comprising administering to a host in need thereof an effective amount of a vector encoding a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
100. A method according to claim 99, for the treatment of cancer.
101. A method according to claim 99, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
102. A method of treatment and/or prevention of a disease resulting from excessive immune responses, comprising administering to a host in need thereof an effective amount of a polypeptide comprising a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
103. A method according to claim 102, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
104. A method for treatment and/or prevention of a disease resulting from excessive immune responses, comprising administering to a host in need thereof an effective amount of a vector encoding a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

105. A method according to claim 104, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.

106. A method of screening for molecules generated by combinatorial chemistry, which inhibit NIK and IL-2 γ chain receptor interaction comprising:
exposing cells expressing NIK, a reporter plasmid encoding luciferase under the control of an NF- κ B inducible promoter (pcDNA3 luciferase) and a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18) or fragments thereof, to individual synthetic organic compounds, testing luciferase expression and isolating compounds able to inhibit or enhance NF- κ B activation.

107. A molecule isolated by the method according to claim 106.

108. A polypeptide comprising the intracellular domain of the IL-2 γ chain receptor, or a mutein, fusion protein, functional derivative, active fraction, circularly permuted derivative or fragment thereof.

109. A polypeptide according to claim 108, comprising the amino acid sequence of SEQ ID NO: 1.

110. A polypeptide according to claim 108, encoded by the nucleotide sequence of SEQ ID NO: 5.

111. A polypeptide according to claim 108, comprising the amino acid sequence of SEQ ID NO: 2.

112. A polypeptide according to claim 108, encoded by the nucleotide sequence of SEQ ID NO: 6.

113. A polypeptide according to claim 108, comprising the amino acid sequence of SEQ ID NO: 3.

114. A polypeptide according to claim 108, comprising the amino acid sequence of SEQ ID NO: 17.

115. A DNA sequence encoding a polypeptide according to anyone of claims 108 to 115.

116. A DNA sequence according claim 115, wherein the DNA sequence further encodes a signal peptide.
117. A DNA according to claim 115, wherein the signal peptide sequence is that of the growth hormone.
118. A polypeptide comprising a fragment of NIK, corresponding to the cyc binding domain (SEQ ID NO:18), or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
119. A DNA sequence encoding a polypeptide according to claim 118.
120. A DNA according claim 119, wherein the DNA further encodes a signal peptide sequence.
121. A DNA according to claim 119, wherein the signal peptide sequence is that of the growth hormone.
122. A vector comprising a DNA according to anyone of claims 115 to 121.
123. A host cell harbouring a vector according to claim 122 capable of expressing a polypeptide according to anyone of claims 108 to 114.
124. A host cell according to claim 123, wherein the host cell is prokaryotic.
125. A host cell according to claim 123, wherein the host cell is eukaryotic.
126. A host cell according to claim 125, wherein the host is a CHO cell.
127. A method for producing a polypeptide according to anyone of claims 108 to 114, comprising culturing a host cell according to anyone of claims 123 to 126 and isolating the polypeptide produced.
128. An intrabody reacting with 44MPD or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
129. An intrabody reacting with 41MDD or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.
130. An intrabody reacting with NIK640-720 or a mutein, fusion protein, functional derivative, circularly permuted derivative or fragment thereof.

131. The use of an intrabody according to anyone of claims 128 to 130, in the manufacture of a medicament for the treatment or prevention of a disease in which NIK activation is involved in its pathogenesis.
132. The use according to claim 131, for the treatment of cancer.
133. The use according to claim 131, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
134. The use of an intrabody according to claim 130, in the manufacture of a medicament for the treatment or prevention of a disease, wherein induction by a cytokine, having the common gamma chain in its receptor is involved in the pathogenesis of the disease.
135. A method for the treatment or prevention of a disease in which NIK activation is involved in its pathogenesis, comprising the administration of an effective amount of an intrabody according to anyone of claims 128 to 130.
136. A method according to claim 135, for the treatment of cancer.
137. A method according to claim 135, for the treatment of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiac infarct, Alzheimer's disease, or atherosclerosis.
138. A method of treatment and/or prevention of a disease in which activation of a cytokine, having the common gamma chain in its receptor, is involved in the pathogenesis of the disease, comprising administering to a host in need thereof an effective amount of an intrabody according to claim 130.

For the applicant

Henry Einav

Figure 1

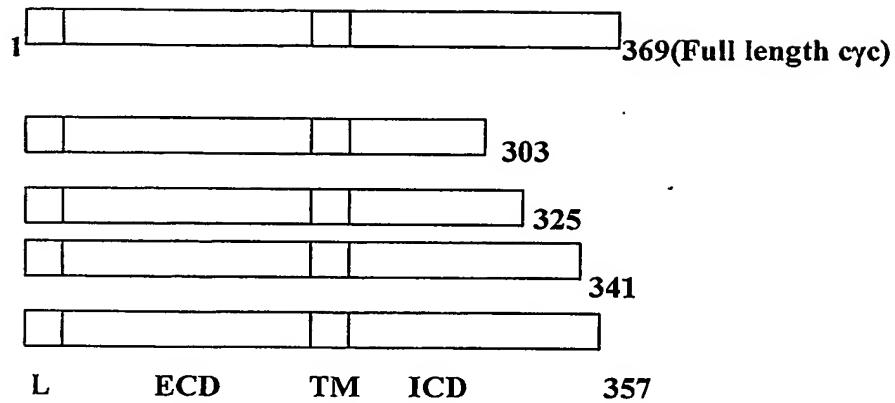


Figure 2

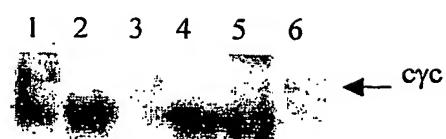


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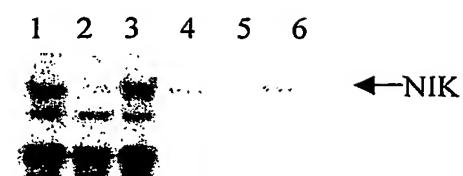


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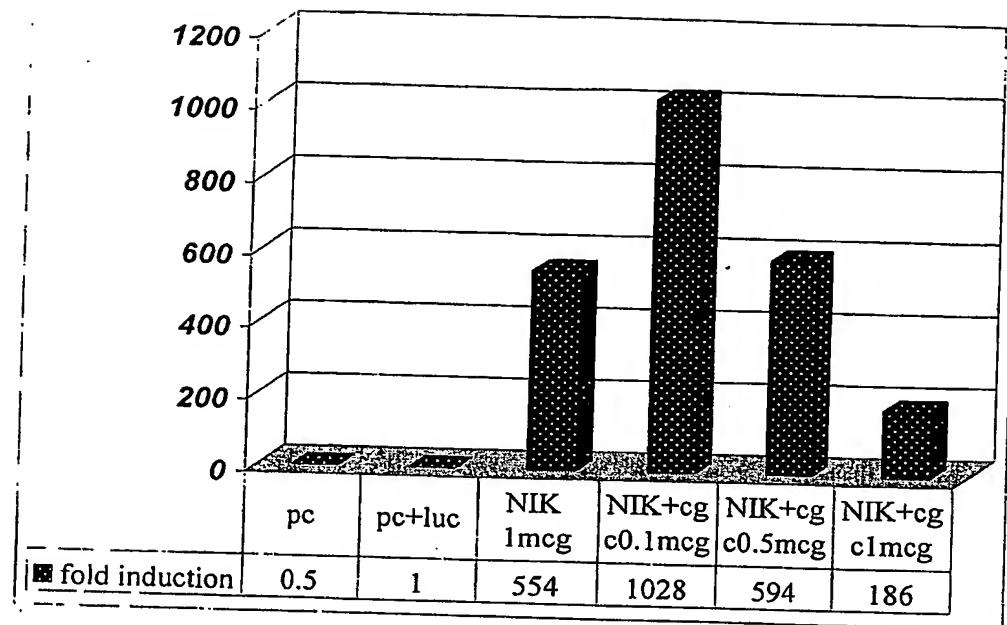


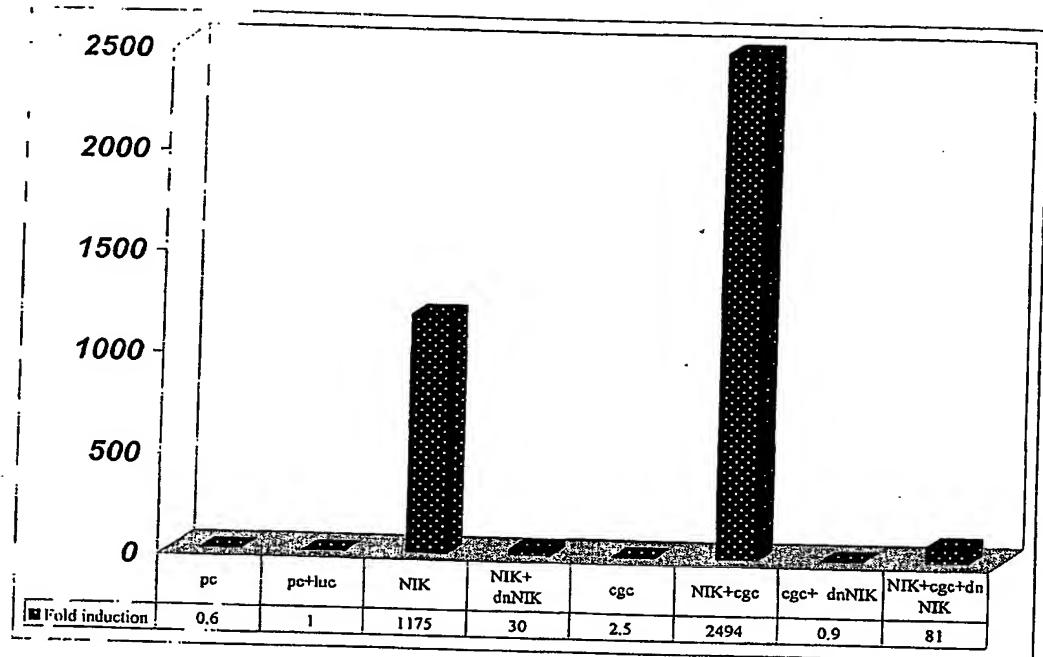
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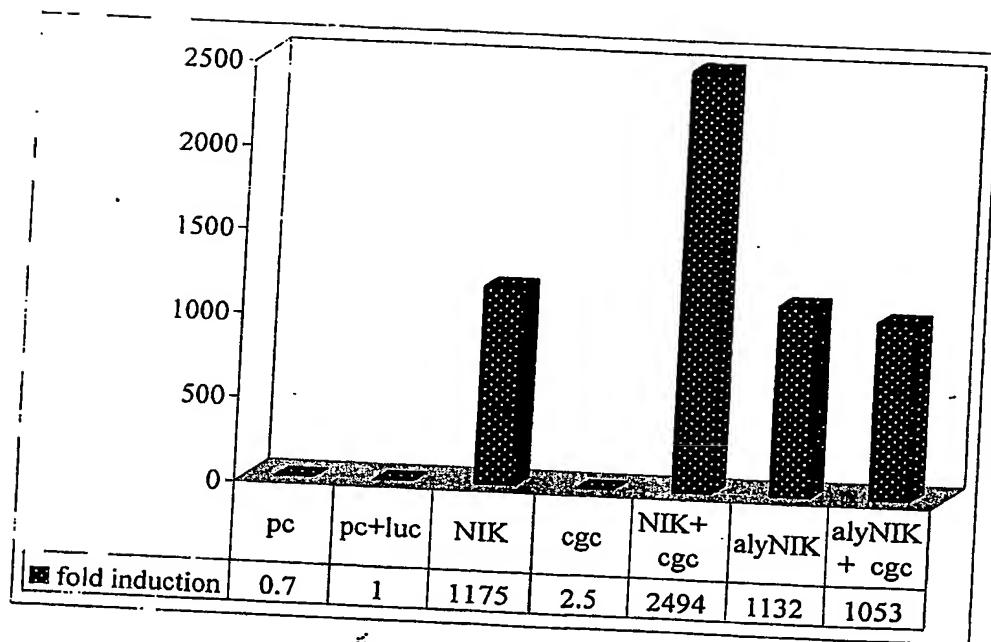
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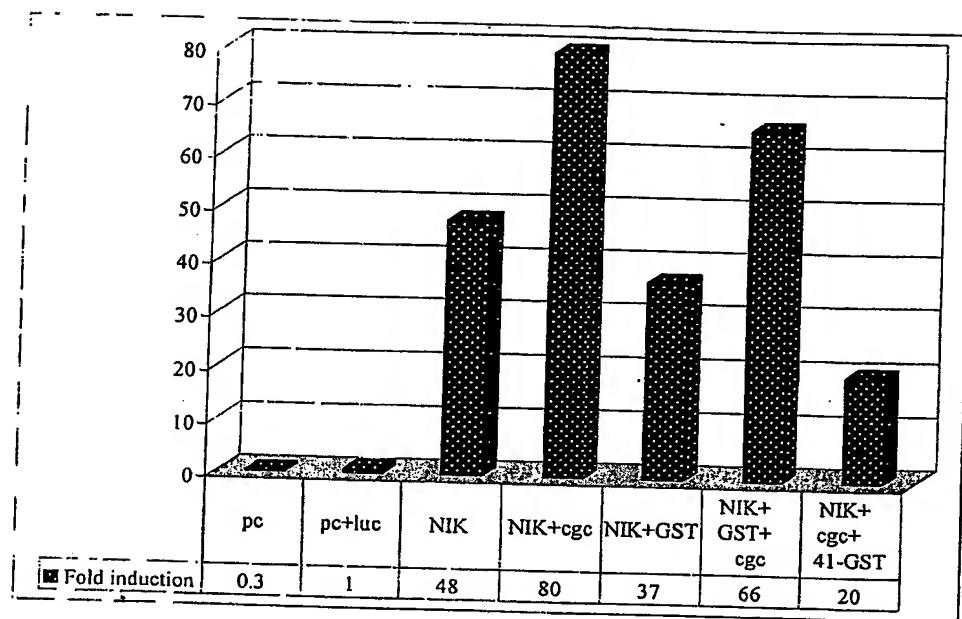
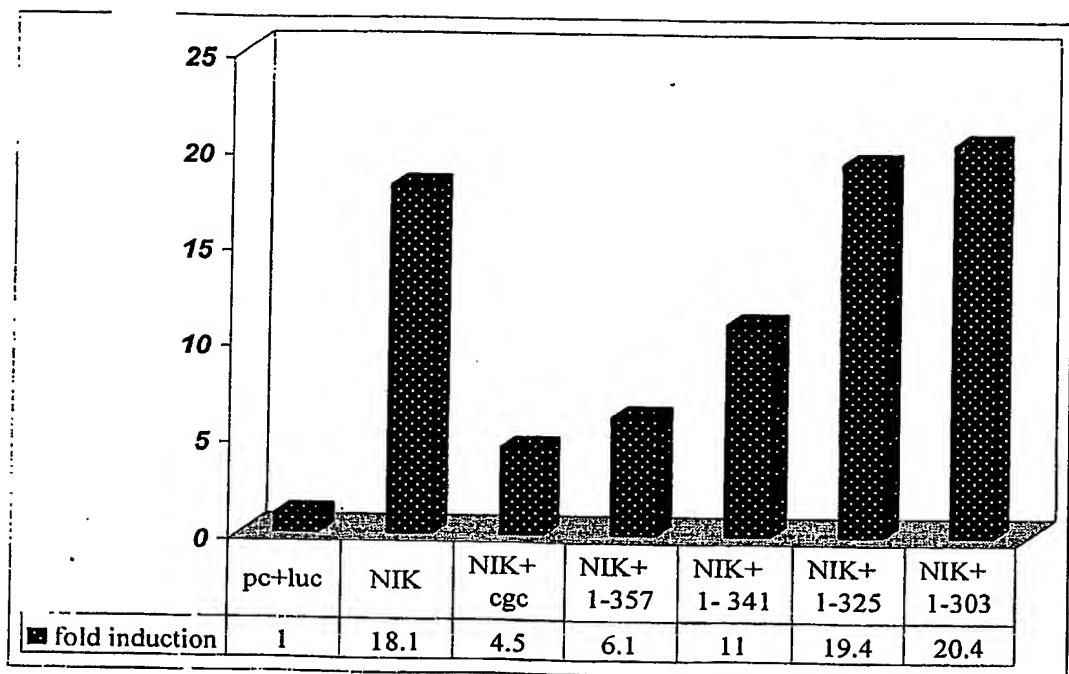
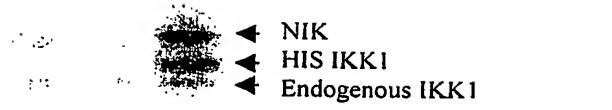
Figure 7

Figure 8



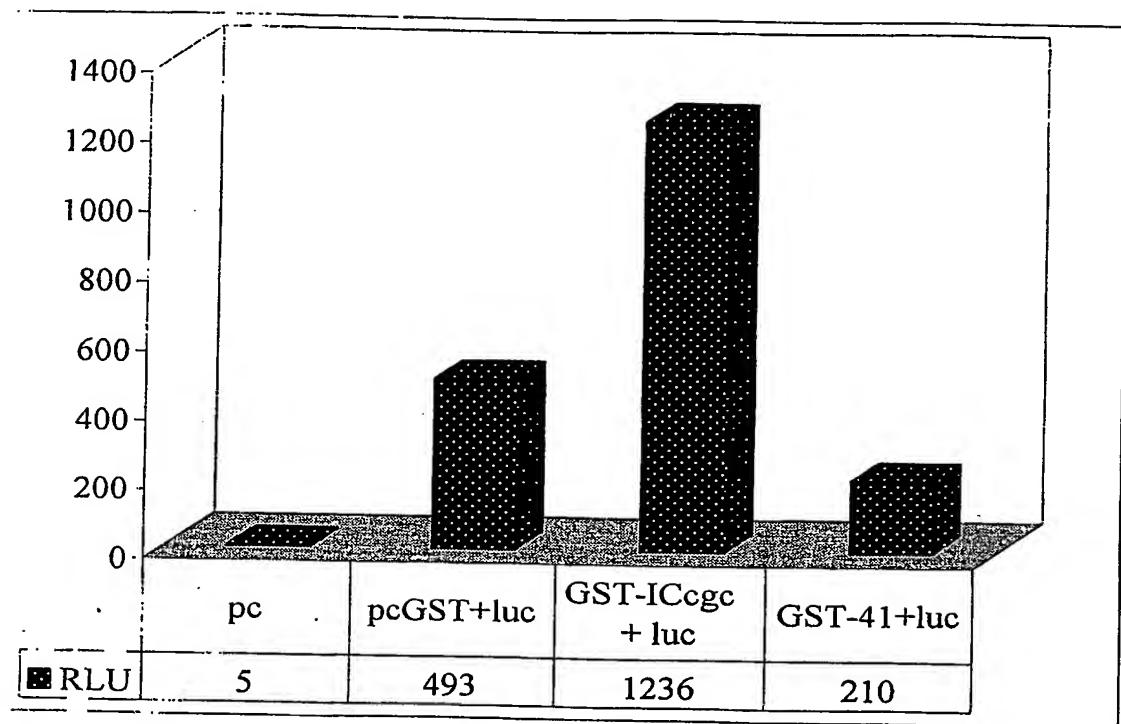
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Figure 9



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Ref: 530A

Figure 10



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Figure 11

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Figure 12

LCLVSEIPPKGALGEGPGASPCNQHSPYWAPPCYTLKPET

Figure 13

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Figure 14

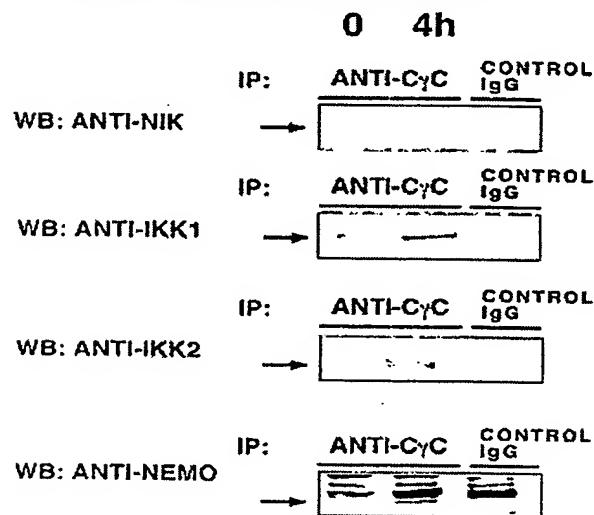
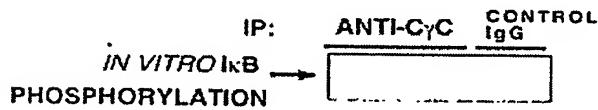
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Figure 15

WAPPCYTLKPET

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Figure 16 A and B**CYTOKINE-INDUCED IKK-RECRUITMENT TO THE C_γC AND ACTIVATION****A-****PBMC, IL2 TREATMENT****B-**

530a.ST25
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wallach, David

Ramakrishnan, Parameswaran

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Page 5

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25 530a.ST25

30

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Glu

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